Homology Modeling of the Transmembrane Domain of the Human Calcium Sensing Receptor and Localization of an Allosteric Binding Site*

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A homology model for the human calcium sensing receptor (hCaR) transmembrane domain utilizing bovine rhodopsin (bRho) structural information was derived and tested by docking the allosteric antagonist, NPS 2143, followed by mutagenesis of predicted contact sites. Mutation of residues Phe-668 (helix II), Arg-680, or Phe-684 (helix III) to Ala (or Val or Leu) and Glu-837 (helix VII) to Ile (or Gln) reduced the inhibitory effects of NPS 2143 on [Ca$^{2+}$]$^i$ responses. The calcimimetic NPS R-568 increases the potency of Ca$^{2+}$ in functional assays of CaR. Mutations at Phe-668, Phe-684, or Glu-837 attenuated the effects of this compound, but mutations at Arg-680 had no effect. In all cases, mutant CaRs responded normally to Ca$^{2+}$ or phenylalanine, which act at distinct site(s). Discrimination by the Arg-680 mutant is consistent with the structural differences between NPS 2143, which contains an alkyl bridge hydroxyl group, and NPS R-568, which does not. The homology model of the CaR transmembrane domain robustly accounts for binding of both an allosteric antagonist and agonist, which share a common site, and provides a basis for the development of more specific and/or potent allosteric modulators of CaR. These studies suggest that the bRho backbone can be used as a starting point for homology modeling of even distantly related G protein-coupled receptors and provide a rational framework for investigation of the contributions of the transmembrane domain to CaR function.

The calcium sensing receptor (CaR) is a member of family C of the G protein-coupled receptor (GPCR) superfamily, which includes metabotropic glutamate receptors (mGluRs), γ-aminobutyric acid receptors (GABA$_B$Rs), and a large family of putative pheromone and taste receptors. In addition to the seven transmembrane helices, which are the signature characteristic of all GPCRs, members of this family have large extracellular domains (with structural homology to bacterial periplasmic binding proteins) that contain the agonist binding site(s) (1). CaR signaling includes G$_q$-mediated activation of phosphatidylinositols, production of inositol 1,4,5-trisphosphate and diacylglycerol, followed by increases in intracellular Ca$^{2+}$ in all cell types examined, activation of G$_q$-mediated pathways in some cell types (reviewed in Ref. 2), and, through an interaction of the CaR carboxyl terminus with filamin A, activation of the mitogen-activated protein kinase cascade (3, 4).

CaR not only binds and is activated by Ca$^{2+}$ at its agonist binding site (localized to the amino-terminal 500 amino acids) (5), but also interacts with allosteric modulators via several sites that have been shown to be distinct from the agonist binding site. CaR activity (in the presence of Ca$^{2+}$) is allosterically modulated by amino acids (6), small peptides (7, 8), as well as a family of structurally related phenylalkylamines (9, 10). The phenylalkylamines are of particular interest, because both allosteric agonists (calcimimetics) and antagonists (calcilytics) have been identified, typified by NPS R-467 or R-568 (calcimimetics) (9) and NPS 2143 (calcilytic) (11). The putative binding site(s) for amino acids and potentially for peptides (including poly-L-arginine, protamine, β-amylloid) have been identified within the extracellular agonist binding domain by homology with metabotropic glutamate receptors, and have been localized to a tripeptide motif, Ser-169–171 (12). Mutant CaRs, which are no longer modulated by amino acids, still exhibit altered potency of Ca$^{2+}$ in the presence of phenylalkylamines (13), confirming that the phenylalkylamines interact at a distinct site. Chimeras between CaR and mGluRs have shown that the phenylalkylamine binding site is localized to the transmembrane domain of CaR (13–15), and recent studies have suggested that negatively charged residues within the e2 and e3 loops may contribute to the binding of NPS R-568 (15, 16). Identification and characterization of the phenylalkylamine binding site on CaR might provide a basis for development of more specific compounds capable of modulating CaR activity on the background of the relatively constant extracellular Ca$^{2+}$ concentrations observed in vivo.

With one high resolution crystal structure of a GPCR available (bovine rhodopsin bRho (17)), any homology modeling of GPCRs based on experimental structural information remains speculative and is only useful in combination with reliable experimental data. Implications of the structure of bRho on
generating models for other GPCRs are addressed by Balles- 
teros and Palczewski (Ref. 18). Various attempts to build and 
refine atomic-level GPCR models from structural information 
(for example, see reviews in Refs. 19–22) and/or first principles 
(e.g. 23, 24) have been reported. In this work, we have opted for 
the pragmatic assumption that the backbone of bRho is a 
reasonable starting point for building a model of the TTM 
region of human CaR, despite the fact that CaR is not a mem-
ber of the rhodopsin family of GPCRs. Furthermore, distinc-
tions between active or inactive morphologies in the structure 
were not incorporated into the model. We have assumed that 
the ligands of interest interact primarily with the helical trans-
membrane (TTM) parts of the receptor and refrained from 
building the extra- and intracellular loops (except for extracel-
lar loop 1, connecting helices II and III, see below and in 
“Experimental Procedures”). Here we report the general 
features of the model for the transmembrane domain of human 
CaR, as well as the identification of residues predicted to con-
tact the phenylalkylamines NPS 2143 and NPS R-568. Mutations 
at the predicted residues eliminate or severely attenuate 
efficacy of the allosteric compounds, whereas the abilities of 
Ca2+ or the amino acid phenylalanine to activate the receptor 
are largely unaffected. In addition to providing a basis for 
development of more specific and/or potent allosteric modula-
tors of CaR, these studies provide an example of the general-
izability of the bRho backbone as a starting point for homology 
modeling of even distantly related GPCRs.

EXPERIMENTAL PROCEDURES

Generation of the Homology Model—The helical parts of bRho were 
isolated from the x-ray coordinates (protein data base file 1F88.pdb) and 
used as a template. The selection was moderately extended on both 
ends of the helices. The final sequence fragments of bRho chosen to 
serve as a template for model building are those listed in Table I. A 
specific routine was used to perform the sequence alignment of the TTM 
domain of human CaR to the selected residues of the corresponding 
regions in bRho, taking into account that no insertions or deletions can 
be applied for the superposition of helices. In addition to the bRho 
sequence, the consensus sequence of the entire rhodopsin family was 
used in this procedure. The alignment was optimized with regard to 
various physical properties of the amino acids such as their formal 
charge, hydrophobicity, and size of side chains. Once a given sequence 
alignment was established, the side chains in bRho were replaced by 
the corresponding ones of CaR, using version 2.9 of the SCWRL 
program (25, 26) with the side-chain conformation library bRho.bcl. 
Jul.sorlib. The resulting structure was refined by molecular mechan-
ics, using the parm94 parameter set of AMBER (27) with the conjugate 
gradient minimizer as implemented in our in-house software WitP.

The energy minimization was carried out with all Ca atoms being fixed 
at this stage. Electrostatics were evaluated with the original force field 
charges, using a distance-dependent dielectric function with ε = 1 + r. 
Helices II and III (presumed shorter than in bRho, according to the 
sequence alignment in Table I) were then connected to form the extra-
cellular loop 1, and the final structure was again refined, this time 
allowing all the residues connecting helices II and III to move freely 
without any constraints.

The ligand NPS 2143 was assigned standard AMBER atom types 
resulting in acceptable force field parameters for this type of structure. 
Partial charges were computed with the MPEOE method in the WitP 
software. These charges are close to the RESP charges to be expected 
for such connectivities and were considered as suitable for the current 
work. Docking of the ligand into the receptor was carried out exactly 
without any constraints.

The starting point for docking was the presumed close interaction 
between Glu-837 and the charged nitrogen on the ligand. The rest of 
the ligand was then positioned by avoiding severe steric overlap with 
the receptor, trying to embed the aromatic (i.e. hydrophobic) groups as 
deeply as possible into the receptor while respecting the internal strain 
of the ligand. The eventual refinement of the entire complex was again 
carried out, having generated gradient minimized with the minimizer 
as for the receptor alone, but this time allowing all Ca in the receptor 
to move freely within 0.5 Å, applying a harmonic force of 10 kcal/molÅ2 
beyond that distance (“tethering”). The resulting geometry is essen-
tially free of strain in the framework of the applied force field. The 
docking of NPS R-568 into CaR was performed in the same way as for 
NPS 2143, however, the docking mode for the latter was used as the 
starting point.

Mutagenesis and Cell Transfections—Point mutations were produced 
in the CaR-EoFP background (fusion protein previously described in 
Ref. 28) by site-directed PCR mutagenesis and confirmed by restriction 
endonuclease digestion and direct sequencing. Sequences of primer 
pairs used for mutagenesis are available upon request. HEK-293 cells 
(American Type Culture Collection, Rockville, MD) were grown in high 
glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Grand Island, 
NY), supplemented with 10% fetal bovine serum (Invitrogen), penicillin 
(50 units/ml), streptomycin (50 μg/ml), and amphotericin B (2 μg/ml) at 
37 °C, 5% CO2. For transient transfections, 1 μg of each CaR construct, 
2 μl of NovaFECTO (Venn Nova LLC, Pompano, FL), and 100 μl of 
medium were premixed, then added to HEK-293 cells in a 24-well plate, 
supplemented with regular medium as previously described (28). Ex-
periments were carried out 68–72 h after transfection.

IP Formation Assay—Transiently transfected HEK-293 cells were 
seeded to confluence in 24-well plates and maintained for 1 day in full 
medium before labeling with myo[3H]inositol (100 MBq/ml, ART/Anawa 
Trading, Wangen, Switzerland) for 24 h in serum-free Dulbecco’s modi-
fied Eagle’s medium. Cells were then washed and incubated at 37 °C in 
a phosphate-free physiologic salt solution, and experiments were 
carried out exactly as previously described (45).

Enzyme-linked Immunossay to Quantitate CaR Surface Localization— 
Transiently transfected cells were replated onto 96-well poly-l-
lysine-precoated plates (BD Biosciences, Bedford, MA) after 24 h. Cells 
were fixed 68–72 h after transfection in 4% paraformaldehyde (30 min) 
and blocked with 5% fetal bovine serum in phosphate-buffered saline 
for 60 min (at room temperature). Subsequently, cells were incubated 
with 100 μl of a 1:500 dilution of the primary antibody (polyclonal 
antibody produced against an amino-terminal epitope (LRG, residues 
374–391, first described by Goldsmith et al. (29), Genemed Synthesis, 
Inc., San Francisco, CA) for 60 min, then with 100 μl of a 1:1000 dilution 
of the secondary antibody (horse radish peroxidase-conjugated anti-rab-
bit IgG, Amersham Biosciences, Piscataway, NJ). The 3,3’,5,5’-tetra-
methybenzidine liquid substrate system (Sigma, St. Louis, MO) was 
used to develop the plate; reactions were halted with 1 μl H2SO4. 
The plate was analyzed on a PowerWave345 microplate scanning spectrophotometer at 450 nm (BIOTEK Instruments, Inc., Winooski, VT).

Measurement of Intracellular Ca2+—Single cell fluorescence 
measurements of [Ca2+]i were made as previously described (28, 32, 33). 
Solutions containing the calcilytic NPS 2143 N-(3R)-2-hydroxy-3-(2-
cyano-3-chlorophenoxo)propy1-1,1-dimethyl-3-(2-naph-
thylyethylamine. B, calciminetic NPS R-568, (R)-N-(3-methoxy-a-phenyl-
ethyl)-3’(2’-chlorophenyl)-1-propylamine hydrochloride.

FIG. 1. Compound structures. A, calcilytic NPS 2143, N-(3R)-2-
hydroxy-3-(2-cyano-3-chlorophenoxo)propy1-1,1-dimethyl-3-(2-nap-
thyl)ethy1amine. B, calciminetic NPS R-568, (R)-N-(3-methoxy-a-phenyl-
ethyl)-3’(2’-chlorophenyl)-1-propylamine hydrochloride.
carried out at room temperature (22–24 °C). 10–20 cells were analyzed during each experiment; experiments were repeated in at least three independent transfections unless otherwise noted. Selected regions of cells were excited at 340/380 nm and emitted light collected at 510 nm at intervals of 10 s using an imaging system (Universal Imaging Corp., West Chester, PA) based on the MetaFluor software package. Background images were obtained at the beginning of each experiment from an area devoid of cells. Calibration of [Ca²⁺], was done with a series of buffered calcium standards (Molecular Probes Inc., Eugene, OR) assuming a Kᵣ of 145 nM determined in vitro at 22 °C (30).

Data Analysis—Average [Ca²⁺], in a given condition was calculated as the average of at least 10 consecutive time points for each cell analyzed. For studies of intracellular Ca²⁺ oscillations, a cell was characterized as exhibiting oscillations if three or more consecutive peaks could be clearly differentiated, and the amplitude of the peaks was > 10 nM [Ca²⁺]. Data were fitted to the Hill equation by least squares minimization using the Marquardt-Levenberg algorithm (NFIT, Island Software). Back-ground images were obtained at the beginning of each experiment from an area devoid of cells. Calibration of [Ca²⁺], was done with a series of buffered calcium standards (Molecular Probes Inc., Eugene, OR) assuming a Kᵣ of 145 nM determined in vitro at 22 °C (30).

RESULTS

Sequence Alignment of hCaR with Bosine Rhodopsin—The alignment shown in Table I is the result of an iterative process following several rounds of model building and experimental verification or falsification. For all helices, the global location in the sequence is consistent with earlier estimates based on hydrophobicity analysis. Possible alignments with a good overall score (see “Experimental Procedures”) were taken as a starting point; the final alignment emanates from additional information provided by the mutational studies. The very low homology of the 7TM domain of human CaR to that of bRh (the template) or to the rhodopsin family consensus, and the absence of various key residues usually conserved in the rhodopsin family, did not allow the straightforward use of standard sequence alignment tools. Nevertheless, the alignment algorithm that was used worked well for helices I, III, V, VI, and VII. Difficulties were encountered particularly for helix IV, and the alignment in Table I was ultimately chosen based on the proline doublet toward the C-terminal of the helix. Note that helix IV is not involved in any direct interactions with the ligands considered here, and experimental data challenging this alignment are therefore not available. Similar problems were encountered for helix II. In this case, however, the mutational studies demonstrating the importance of Phe-668 over Phe-667 in ligand binding provided critical evidence in favor of the alignment shown.

Building the Model and Docking the Ligands—Selection of residues to be mutated was originally based on a model for human CaR, which was built using the Baldwin Ca trace of rhodopsin (31). Glu-837 at helix VII was selected as the main anchor point for binding of NPS 2143, and the ligand was embedded as described under “Experimental Procedures”; mutational studies concentrated on amino acids that were within a distance of 6 Å from the ligand in the preliminary 3-dimensional model, preferentially considering residues within the helices rather than within the loops. The primary results from the mutations were then incorporated into the refinement of the model of the CaR-NPS 2143 complex, which also resulted in refinement of the sequence alignment. Fig. 2A illustrates the predicted location of the allosteric modulator site at the extracellular transmembrane interface. Fig. 2B shows the results of docking trials using the calcilytic NPS 2143 and indicates the residues predicted to participate in bonding interactions with the ligand. The final model indicates that if the carboxylic group of Glu-837 forms a salt bridge with the protonated amino group in NPS 2143, then Phe-668 and Phe-684 may form hydrophobic interactions with the planar rings of the ligand. Additional interactions are suggested with Arg-680. As mentioned above, in earlier models based on different possible sequence alignments, it was also possible that Phe-667 might interact with the ligand. The final results from mutation studies (see below) indicate that this residue is not involved in the binding of the ligand, supporting the model presented here.

Initial Characterization of Predicted Ligand Interaction Sites—The initial model predicted potential interaction(s) of NPS 2143 with Phe-667 or Phe-668 in helix II, Arg-680 and Phe-684 in helix III, and Glu-837 in helix VII at the extracellular interface. Point mutations at each site were generated, with substitutions of Ala, Leu, and Val for each phenylalanine, and Ile and Gln for Glu-837. To screen out mutants that were inefficiently trafficked to the plasma membrane, we performed ELISA assays utilizing paraformaldehyde-fixed, non-permeabilized cells. The data, presented in Fig. 3, indicate that most mutants were efficiently trafficked to the plasma membrane; exceptions were the F668A, F668L, F668V and R680L, R680V mutants, which are present at ≤40% of wt CaR levels. As a preliminary screening of mutants for functional activity, HEK-293 cells were transiently transfected with wild type CaR-EGFP or a mutated CaR and IP formation was measured. A measure of the general functionality of the mutants was obtained from the responses to a range of extracellular Ca²⁺ concentrations (2, 5, and 8 mM) (Fig. 4A), the relative potency of the allosteric antagonist NPS 2143 (at 0.1, 1, and 10 μM) was determined in the presence of 8 mM Ca²⁺ (Fig. 4B), and the potency of the allosteric agonist NPS R-568 (at 1 and 10 μM) was determined in the presence of 2 mM Ca²⁺ (Fig. 4C). Wild type CaR is activated in a concentration-dependent manner by extracellular-
NPS 2143 causes a progressive decrease in IP formation at 8 mM Ca\(^2+\)/H\(11001\), whereas NPS R-568 causes a dramatic increase in IP formation at 2 mM Ca\(^2+\)/H\(11001\). Mutations at position Phe-667 had activation characteristics comparable to wild type CaR (Table II), whereas the adjacent position Phe-668 exhibits normal activation by Ca\(^2+\)/H\(11001\) but attenuated responses to either NPS 2143 or NPS R-568 (Fig. 4, A–C). Mutations at positions Phe-684 and Glu-837 also exhibit attenuated responses to both allosteric ligands. Finally, mutations at position Arg-680 attenuate responses to NPS 2143 but have no effect on activation by extracellular Ca\(^2+\)/H\(11001\) and NPS R-568 relative to wt CaR (Fig. 4).

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**Fig. 2.** Model for NPS 2143 binding to human CaR. A, location of NPS 2143 with respect to the seven transmembrane helices of CaR. Model was generated as discussed under “Experimental Procedures.” B, close-up view of hCaR residues (shown in ball and stick representation, with enclosing transparent molecular surface), which contribute to the binding of NPS 2143 (shown as a Corey-Pauling-Koltun space-filling model). The predominant interaction is the salt bridge to Glu-837 via the charged nitrogen in the ligand. Arg-680 can interact with the -OH group in the ligand, and Phe-668 and Phe-684 make close hydrophobic contacts and \(\pi\)-stacking with ligand rings. For both panels A and B, roman numerals indicate helix number.

**Fig. 3.** Plasma membrane localization of wild type CaR and various point mutants. ELISA assay of wt CaR-EGFP and CaR-EGFP bearing individual point mutations at the locations indicated; the horizontal dashed line denotes the signal derived from untransfected HEK-293 cells (nonspecific signal). Bars represent means \pm S.E. obtained from at least three independent transfections. Plasma membrane localization is characterized in arbitrary absorbance units (A.U.).

**Fig. 4.** IP formation of wt CaR and receptors with point mutations at predicted NPS 2143 contact sites. Experiments were performed as described under “Experimental Procedures.” For all panels: closed circles, wt CaR; open circles, F668A; closed triangles, R680A; open triangles, F684A; closed squares, E837I. A, IP formation in response to extracellular Ca\(^2+\) (0.5–10 mM) expressed in dpm \times 10^{-3}. B, inhibition of IP formation by NPS 2143 (0.1, 1, or 10 \(\mu\)) in the constant presence of 8 mM extracellular Ca\(^2+\). Normalized IP formation (%) = (IP formation in NPS 2143 – IP formation in 0.5 mM Ca\(^2+\))/IP formation in 0 mM Ca\(^2+\) \times 100. C, enhancement of IP formation by NPS R-568 (1 and 10 \(\mu\)) in the constant presence of 2 mM extracellular Ca\(^2+\). Normalized IP formation (%) = (IP formation in NPS R-568)/IP formation in 2 mM Ca\(^2+\) \times 100.
intracellular Ca\(^{2+}\) (32, 33), in particular with regard to interactions of the receptor with allosteric activators (32). Cells expressing comparable levels of wt or mutant CaR can be selected based upon GFP fluorescence intensity (all constructs are fused to EGFP at the carboxyl terminus), and receptor responses can be assessed with high time resolution. Therefore, to characterize the differences in sensitivity of the mutants identified in the preliminary screen (IP formation, Fig. 4) to the allosteric modulators, all mutants were analyzed for their ability to modulate intracellular Ca\(^{2+}\). Fig. 5 illustrates the dose-response relations for extracellular Ca\(^{2+}\)-mediated activation of CaR, monitored by changes in intracellular Ca\(^{2+}\). All receptor mutants were activated in a cooperative manner by increases in extracellular Ca\(^{2+}\), although several of the mutants exhibited modest changes in the EC\(_{50}\) for Ca\(^{2+}\) and/or the Hill coefficient (Fig. 5 for mutations of key residues to alanine, and Table II for all substitutions). The reduced maximal responses observed for several of the mutants (F668A and R680A) correlated with the reduced plasma membrane localization determined by ELISA assay, whereas for others (F684A and E837I) the reduction in maximal response was observed despite enhanced surface localization (Fig. 3).

To determine whether point mutations of the amino acid residues proposed for the allosteric binding site (Phe-668, Phe-684, Arg-680, and Glu-837) decreased the potency of NPS 2143 for inhibition of [Ca\(^{2+}\)], responses, transiently transfected HEK-293 cells were briefly exposed to 5 mM [Ca\(^{2+}\)], to activate CaR, then increasing concentrations of NPS 2143 (0.1, 1, and 10 \(\mu M\)) were added for 2–4 min each (Fig. 6A) (exposure times were longer for mutant CaRs to ensure detection of potentially slower responses). A significant reduction of average [Ca\(^{2+}\)], and a cessation of intracellular Ca\(^{2+}\) oscillations was observed for 1 and 10 \(\mu M\) NPS 2143 in cells transfected with wild type CaR (cells oscillating under control conditions, 90.0%; cells oscillating in 10 \(\mu M\) NPS 2143, 7.7%, \(p < 0.01\), Fig. 6B). For cells transfected with the point mutations having potential contacts with NPS 2143, the responses to NPS 2143 were significantly attenuated. Mutation F668A exhibited a decrease in average [Ca\(^{2+}\)], at all concentrations of NPS 2143, and a decrease in the percentage of cells exhibiting intracellular Ca\(^{2+}\) oscillations at 10 \(\mu M\) NPS 2143 (control, 67.9%; NPS 2143, 38.1%, \(p < 0.05\), Fig. 6B). The mutants R680A, F684A, and E837I showed no significant decrease in average [Ca\(^{2+}\)], nor a decline in the percentage of cells exhibiting intracellular Ca\(^{2+}\) oscillations in the presence of even the highest concentration of NPS 2143 (Fig. 6, A and B). In summary, the whole cell [Ca\(^{2+}\)], responses to NPS 2143 (0.1–10 \(\mu M\)) were attenuated for F668A and eliminated in the R680A, F684A, and E837I mutants.

NPS R-568-mediated Increase in Ca\(^{2+}\) Potency Is Dependent upon Phe-668, Phe-684, and Gln-837—The calcimimetics NPS R-568 and NPS R-467 and the calcilytic NPS 2143 are competitive ligands, potentially binding to overlapping site(s) on CaR (11). We therefore examined whether mutations at the key residues involved in binding of NPS 2143 (Phe-668, Arg-680, Phe-684, and Gln-837) also attenuated the effects of the calcimimetic NPS R-568. We have extensively characterized the functional effects of NPS R-568 on wt CaR (32) via measurement of single-cell dose-response relations for extracellular Ca\(^{2+}\), as well as modulation of CaR-mediated intracellular Ca\(^{2+}\) oscillations. Therefore, similar approaches were utilized to characterize NPS R-568 effects on CaR transmembrane domain mutations. HEK-293 cells were transiently transfected with either wt CaR-EGFP or CaR-EGFP point mutants, loaded with fura-2-AM at 2 \(\mu M\) [Ca\(^{2+}\)], and subsequently exposed to increasing concentrations of [Ca\(^{2+}\)], from 0.5 to 10 \(\mu M\) in the absence or presence of 1 \(\mu M\) NPS 568. The characteristic decrease in the EC\(_{50}\) for extracellular Ca\(^{2+}\) produced by NPS R-568 in cells transfected with CaR-EGFP (EC\(_{50}\) (Ca\(^{2+}\)) = 3.3 ± 0.05 mM; EC\(_{50}\) (Ca\(^{2+}\) + 1 \(\mu M\) NPS 568) = 2.0 ± 0.06 mM) was abolished in cells transfected with receptors bearing the F668A, E837I, or E837Q mutations (Fig. 7 and Table II). As seen with NPS 2143, the NPS R-568 potency for the F667A mutant was comparable to wt CaR-EGFP (Fig. 7, analysis in Table II), confirming the importance of the neighboring Phe-

### Table II

| Construct     | EC\(_{50}\) ± S.D. (nM) | n ± S.D. | R\(_{max}\) ± S.D. (nM) |
|---------------|---------------------------|---------|-------------------------|
| CaR-EGFP      | 3.3 ± 0.05                | 1.13 ± 0.14 | 188.59 ± 1.82 |
| F667A         | 2.95 ± 0.04               | 2.75 ± 0.09 | 207.44 ± 1.6 |
| F668A         | 3.69 ± 0.01               | 2.5 ± 0.14 | 162.51 ± 2.39 |
| F668L         | 2.67 ± 0.13               | 3.71 ± 0.55 | 145.82 ± 3.47 |
| E680A         | 4.67 ± 0.68               | 1.89 ± 0.33 | 225.51 ± 17.25 |
| F684A         | 3.8 ± 0.18                | 3.83 ± 0.67 | 103.99 ± 2.32 |
| F684L         | 6.91 ± 1.91               | 1.81 ± 0.34 | 131.79 ± 14.48 |
| E837I         | 3.36 ± 0.26               | 2.49 ± 0.51 | 142.85 ± 5.48 |
| E837Q         | 3.67 ± 0.55               | 2.14 ± 0.53 | 134.75 ± 10.88 |

| Construct     | EC\(_{50}\) ± S.D. (nM) | n ± S.D. | R\(_{max}\) ± S.D. (nM) |
|---------------|---------------------------|---------|-------------------------|
| F668A         | 2.0 ± 0.06                | 2.69 ± 0.27 | 176.35 ± 2.28 |
| F667A         | 1.53 ± 0.32               | 3.08 ± 1.92 | 175.08 ± 8.3 |
| F668L         | 3.64 ± 0.44               | 2.03 ± 0.38 | 151.02 ± 8.96 |
| E680A         | 2.1 ± 0.23                | 2.83 ± 1.11 | 148.5 ± 6.92 |
| F684A         | 2.89 ± 0.52               | 2.35 ± 0.58 | 210.83 ± 11.94 |
| F684L         | 2.99 ± 0.24               | 3.54 ± 0.86 | 100.24 ± 2.54 |
| E837I         | 5.06 ± 0.66               | 2.41 ± 0.45 | 114.82 ± 6.16 |
| E837Q         | 5.57 ± 0.82               | 1.66 ± 0.18 | 161.18 ± 11.61 |
|                | 10.23 ± 5.55              | 1.29 ± 0.23 | 239.66 ± 70.04 |

**Fig. 5.** Extracellular Ca\(^{2+}\) dose-response relations for cells transiently transfected with wt CaR and CaR point mutants. Cells were exposed to increasing concentrations of extracellular Ca\(^{2+}\), starting at 0.5 mM. Averaged peak (transient) responses of at least 20 cells from two or more independent transfections were fitted with the Hill equation: \(R = R_{max} + \frac{[\text{Ca}^{2+}]_o}{EC_{50} + [\text{Ca}^{2+}]_o}\), where \(R_{max}\) is the mean [Ca\(^{2+}\)], at 0.5 mM[Ca\(^{2+}\)], and \(n\) is the Hill coefficient. Data are presented as mean values ± S.E. Fit parameters are shown in Table II. Closed circles, wt CaR; open circles, F668A; closed triangles, R680A; open triangles, F684A; closed squares, E837I.
Homology Modeling of the TM Domain of Human CaR

Obtaining high resolution structures of membrane proteins remains a difficult and low yield process, but homology modeling based on available structures has met with considerable success (35, 36). In this report, we have used the high resolution x-ray structure for bRho as the basis for homology modeling of the transmembrane domain of human CaR, a member of a distinct family of GPCRs. To further refine the preliminary model, docking of the allosteric antagonist NPS 2143, known to interact with CaR via its transmembrane domain, was performed. Finally, the critical residues predicted to be in contact with NPS 2143 were mutated and the resultant receptors studied for their ability to be inhibited by NPS 2143 and to be activated by a related allosteric agonist, NPS R-568. Both the allosteric antagonist and agonist are phenylalkylamines, containing an amino group within the alkyl bridge between phenyl groups. The primary interaction of both ligands was proposed to be through a salt bridge interaction with Glu-837 at the extracellular end of helix 7. Subsequent to development of the preliminary model, Glu-837 was identified by Hu et al. (16) as a potential interaction site for NPS R-568 from a mutagenesis screen of extracellular loop acidic residues, confirming the importance of this residue in the model. Hydropathy plot predictions for the beginning and end of helices in CaR were insufficient for alignment purposes; alignments were refined utilizing both the bRho sequence as well as a consensus sequence for the entire rhodopsin family. A recent report utilized a similar approach based on the bRho structure to model the transmembrane domain of mGluR1 and identify an allosteric antagonist binding site (37).

Early sequence alignments with the bRho sequence (plus family consensus sequence) could not distinguish between resi-
idues Phe-667 and Phe-668 as a contributor to the NPS 2143 binding site, but initial mutagenesis screens utilizing the IP formation assay rapidly focused attention on Phe-668 (Phe-667 behaved like wt CaR in all assays). Experimental discrimination between two adjacent phenylalanine residues validates the orientation of helix II in the final model. Other features of the model critical to NPS 2143 binding are Arg-680, which can interact with the alkyl bridge hydroxyl present on NPS 2143 (but not present in NPS R-568), and Phe-684, which interacts with ligand aromatic rings through π-stacking. Experimental results confirm the essential features of the model, i.e. mutation of Glu-837 to either Ile or Gln reduces the ability of NPS 2143 to decrease CaR-mediated steady state [Ca²⁺]ᵢ or halt CaR-induced intracellular Ca²⁺ oscillations. Residues Phe-668 and Phe-684 had diminished responsiveness to NPS 2143 with respect to the CaR-mediated [Ca²⁺]ᵢ inhibition, and as well as reduced sensitivity to NPS 2143 for inhibition of CaR-mediated [Ca²⁺]ᵢ oscillations.

The binding site of the allosteric agonist NPS R-568 is expected to overlap with that of NPS 2143. Because the active and inactive conformations of CaR, which are promoted by the two compounds, respectively, likely differ within the trans-

| Construct       | Percentage of cells with oscillations of [Ca²⁺]ᵢ |
|-----------------|-----------------------------------------------|
|                 | 3.5 mM | 5 mM | 10 μM | 30 μM |
| CaR-EGFP        | 52.9 (63/119) | 38.4 (68/177) | 76.5 (91/119) | 80.8 (143/177) |
| F667A           | N.D.   | N.D. | N.D.  | N.D.  |
| F668A           | 45.9 (45/98) | 42.6 (29/68) | 11.2 (11/98) | 1.5 (1/68) |
| F668L           | 35.1 (40/114) | N.D. | 7.9 (9/114) | N.D.  |
| F668V           | 0 (0/57) | N.D. | 0 (0/57) | N.D.  |
| R680A           | 50 (29/58) | N.D. | 60.3 (35/58) | N.D.  |
| F684A           | 23.6 (25/106) | 75.4 (46/61) | 9.4 (10/106) | 29.5 (18/61) |
| F684L           | 2.6 (2/76) | N.D. | 0 (0/76) | N.D.  |
| F684V           | 1.7 (1/58) | N.D. | 0 (0/58) | N.D.  |
| E837I           | 20.6 (21/102) | 48.7 (38/78) | 0 (0/102) | 1.3 (1/78) |
| E837Q           | 0 (0/100) | 29.9 (21/78) | 2 (2/100) | 0 (0/78) |

* N.D., not determined.
* P < 0.001.
* P < 0.01.
* P < 0.05.
membrane domain, it was of interest to test the effects of NPS 2143 site mutations on the efficacy of NPS R-568. The characteristic increase in potency of extracellular Ca\(^{2+}\) produced by NPS R-568 in wt CaR persisted in the F667A mutant but was significantly reduced in the F668A, F684A, and E837I mutants. Interestingly, R680A exhibited an NPS R-568-mediated increase in potency of [Ca\(^{2+}\)]\(_e\), indicating structure-dependent discrimination at this position between NPS 2143 (which contains a hydroxyl group coordinated with Arg-680 and is affected by mutations at this position) and NPS R-568 (which does not contain a hydroxyl group and is not predicted to interact with Arg-680). Further confirmation of the involvement of residues Phe-668, Phe-684, and Glu-837 in forming the NPS R-568 binding site comes from studies of intracellular Ca\(^{2+}\) oscillations. For each residue, mutations to Ala (Val or Leu) eliminated or severely impaired the ability of NPS R-568 to elicit intracellular Ca\(^{2+}\) oscillations, despite the continued ability of increments of [Ca\(^{2+}\)]\(_e\) or the amino acid phenylalanine to elicit robust oscillations. Again, mutations at Arg-680 did not alter NPS R-568 responses. Fig. 9 illustrates the likely docking mode of NPS R-568 into the allosteric ligand site on CaR. Because NPS R-568 is somewhat smaller, offers less hydrophobic contacts, and is less sterically constrained than NPS 2143, its position within the binding site is not as well defined. Nevertheless, the reduction of NPS R-568 potency in receptors bearing mutations at Phe-668, Phe-684, and Glu-837 strongly argues that NPS R-568 is indeed interacting at this site.

While the manuscript for this report was under revision, Petrel et al. (47) published a model of the calcium sensing receptor that is based on a sequence alignment to rhodopsin that deviates considerably in several helices from the alignment presented in Table I. However, docking of the Calhex 231 structure (Fig. 1 in Ref. 47) into the receptor model presented here can easily be achieved at the site proposed for NPS-2143 (see Fig. 2B). The salt bridge with Glu-837 remains the predominant interaction, Phe-688 and Phe-684 make hydrophobic contacts, whereas there are no strong electrostatic interactions between the ligand and Arg-680 (which binds to an -OH group in NPS-2143, Fig. 2B). Roman numerals indicate helix number.
the experimental data. Given these considerations, the alignment presented in Table I and the resulting model can account for binding of NPS 2143, NPS R-568, as well as Calhex 231, and thus represents a solid starting point for further experimental validation.

Family C of the GPCR superfamily has several unique features, the most striking of which is an extracellular domain where the agonist/competitive antagonist binding site is localized. In addition, many members of this family (CaR, mGluRs, and GABA<sub>B</sub>Rs) have transmembrane domain-localized allosteric sites for agonists and antagonists, which modulate receptor activation in conjunction with the primary agonist. The allosteric sites represent primary drug targets, providing evidence for support in cell culture and IP formation assays. Here, we present a rational framework for investigation of the contributions of the transmembrane domain to CaR function.

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