Expression and Characterization of Inactivating and Activating Mutations in the Human Ca\textsuperscript{2+}-sensing Receptor* 

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Nearly 30 mutations have been identified to date in the coding region of the extracellular calcium-sensing receptor (CaR) that are associated with inherited human hypo- and hypercalcemic disorders. To understand the mechanisms by which the mutations alter the function of the receptor may help to discern the structure-function relationships in terms of ligand-binding and G protein coupling. In the present studies, we transiently expressed eight known CaR mutations in HEK293 cells. The effects of the mutations on extracellular calcium- and gadolinium-elicited increases in the cytosolic calcium concentration were then examined. Seven inactivating mutations, which cause familial hypercalcioric hyperparathyroidism and neonatal severe hyperparathyroidism, show a reduced functional activity of the receptor because they may 1) reduce its affinity for agonists; 2) prevent conversion of the receptor from a putatively immature, high mannose form into the fully glycosylated and biologically active form of the CaR, in addition to lowering its affinity for agonists; or 3) fail to couple the receptor to and/or activate its respective G protein(s). Conversely, one activating mutation, which causes a form of autosomal dominant hypocalcemia, appears to increase the affinity of the receptor for its agonists.

The recently cloned extracellular Ca\textsuperscript{2+} (Ca\textsuperscript{2+})-sensing receptor (CaR) (1) has provided key insights into the pathogenesis of inherited human hypo- and hypercalcemic disorders (2, 3). The receptor, BopCAr (bovine parathyroid Ca\textsuperscript{2+}-sensing receptor) was first isolated from bovine parathyroid using expression cloning in Xenopus laevis oocytes and shows pharmacological properties nearly identical to those of the native receptor in its responses to extracellular divalent cations (i.e. Ca\textsuperscript{2+} and Mg\textsuperscript{2+}), trivalent cations (e.g. Gd\textsuperscript{3+}, and polyamines (e.g. neomycin) (1). Subsequently, cDNAs encoding the human homolog of the same receptor have been cloned from human parathyroid (4) and kidney (5), using a homology-based strategy. The human and bovine receptors share a high degree of homology at the amino acid level (93% identity). Stimulation of the CaR by agonists activates phospholipase C, with resultant increases in inositol phosphates and the cytosolic calcium concentration (Ca\textsuperscript{2+}) (1). The amino acid sequences of the human and bovine receptors deduced from their cDNAs predict proteins of 1078 and 1085 amino acids, respectively. Both contain a large (600 residues) extracellular amino terminus, a seven-transmembrane domain, similar to that in other members of the G protein-coupled receptor superfamily, and a 200-amino acid carboxyl-terminal tail. The only other G protein-coupled receptors with which the CaR shares a significant degree of homology are the metabotropic glutamate receptors (mGlurS), which also feature large, putatively extracellular domains at their amino termini (about 600 amino acids) (6). The extracellular domains of both the mGlurS and the CaR have been proposed to bind their respective agonists (1, 6).

The physiological importance of the receptor in determining the extracellular calcium concentration has been documented by the characterization of human syndromes resulting from activating or inactivating mutations of the CaR, which alter the function of parathyroid and kidney so as to produce hypo- or hypercalcemia, respectively (2, 3). When present in the homozygous state, inactivating mutations cause familial hypercalcioric hyperparathyroidism (FHH), whereas in the homozygous state, they cause neonatal severe hyperparathyroidism (NSHPT). In contrast, activating mutations are responsible for a form of autosomal dominant hypocalcemia (ADH). Both disorders show abnormal Ca\textsuperscript{2+} sensing and/or handling in kidney and parathyroid. However, the mechanisms by which these mutations alter the function of the CaR have not been studied in detail. In this report, we introduced some of known mutations causing either FHH/NSHPT or ADH into a human parathyroid Ca\textsuperscript{2+}-sensing receptor cDNA (called HuPcAR4.0) (2, 3, 7). We then studied the capacities of the resulting mutant receptor proteins to be properly biosynthesized and processed when expressed in human embryonic kidney cells (HEK293) and to mediate increases in Ca\textsuperscript{2+} in response to CaR agonists (e.g. Ca\textsuperscript{2+} and Gd\textsuperscript{3+}).

EXPERIMENTAL PROCEDURES

Reconstruction of the Receptor—Because the FHH/NSHPT and activating mutations discovered to date are distributed throughout the CaR protein, it would greatly facilitate studies on the expression of these
mutations to have a construct of the receptor that is subdivided into cassettes of convenient sizes for site-directed mutagenesis. We have reconstructed the coding region of HuPCaR4.0 and divided it into five segments of about 450 base pairs each as well as one 100-base pair segment by creating five unique restriction sites in the order of BspEI, NheI, AflI, Hpal, and XhoI (see Fig. 2 in “Results”) without changing the predicted amino acid sequence. In the reconstructed receptor (rHuPCaR4.0), the altered codons were incorporated into the 5’ ends of the respective primers for polymerase chain reaction, and the appropriate segments of the reconstructed receptor were amplified using the human CaR sequence vector (HuPCaR) (5) or the cloning vector (pHUPCA4.0). The modified codons for each of the unique restriction sites in their respective primers were flanked at their 3’ ends by the wild type sequence. Moreover, each forward primer carries a KpnI site preceding the unique restriction sites with the respective modified codon, except that the KpnI site in the first primer (i.e. that nearest the 5’ end of the open reading frame) carries an XbaI site. Each reverse primer carries an XbaI site preceding the unique restriction sites, except that the XbaI site in the last primer precedes the complement sequence of the translational stop codon. The polymerase chain reaction products for each segment of the reconstructed receptor were then cloned into the XbaI and XbaI sites of the cloning vector, pBluescript SK (Stratagene) (17). Each construct is designed as a site-directed mutagenesis cassette. The sequences of the six cassettes, which together encode the full coding sequence of the receptor, were confirmed using an Applied Biosystems 373A Automated DNA Sequencer. Finally, the wild type receptor was reconstructed by fusing the six clones via stepwise subcloning.

Site-directed Mutagenesis—Site-directed mutagenesis to produce specific FHH/NSHPT or ADH mutations was performed using the approach described by Kunkel (8). The dUTP-1 ung-1 strain of Escherichia coli, CJ 236, was transformed separately with each relevant mutagenesis cassette. Uracil-containing, single stranded DNA was produced by infecting the cells with the helper phage, VCSM13. The single stranded DNA was then annealed to a mutagenesis primer, which contained the desired nucleotide change encoding a single point mutation flanked on both sides by wild type sequence. The primer was then extended by the entire single stranded DNA and ligated to generate closed circular heteroduplex DNA. DH5α competent cells were transformed with these DNA heteroduplexes, and incorporation of the desired mutation was confirmed in all cases by sequencing the entire cassette.

Transient Expression of the Reconstructed HuPCaR4.0 (rHuPCaR4.0) and Mutated Receptors Carrying FHH/NSHPT or ADH Mutations in a Human Embryonic Kidney Cell Line (HEK293)—The full-length rHuPCaR4.0 and mutant receptors were cloned into the KpnI and XbaI sites of the mammalian expression vector, pcDNA3 (Invitrogen). The DNA for transfection was prepared using the Midi Plasmid kit (Stratagene). The HEK293 cells used for transfection were grown in 100-mm culture plates. The plates were rinsed twice with PBS and treated with 0.02% EDTA in PBS at 37 °C for 5 min. The detached cells were pelleted and suspended in 300 μl of homogenization buffer (50 mM Tris-HCl, pH 7.4, containing 0.32 mM sucrose, 1 mM EDTA, and protease inhibitors, including 83 μg/ml apro- tinin, 30 μg/ml leupeptin, 1 mg/ml Pefabloc SC, 50 μg/ml calf pancre- atic trypsin inhibitor, 5 μg/ml bestatin, and 5 μg/ml pepstatin) and 1 mM EDTA), and the cells were homogenized with 10 strokes of a motor-driven Teflon pestle in a tightly fitted glass tube. The homogenate was sedimented at 18,800 × g for 20 min to remove nuclei and mitochondria. The super- natant was subsequently sedimented at 43,500 × g for 20 min to pellet the plasma membranes, and the resultant pellet was solubilized with 1% Triton X-100. All steps were carried out at 4 °C. One bovine para- thyroid gland was first cut into small pieces and placed in 600 μl of the above homogenization buffer and then homogenized with 45 strokes in a motor-driven Teflon pestle in a tightly fitted glass tube. The crude plasma membranes were isolated from the homogenate as described above.

Digestion of CaR with Endoglycosidase H or with Peptide-N-Glycosidase F (PNGase F)—For digestion with endoglycosidase H, crude plasma membrane protein, 1.6 μg was first denatured at 37 °C in the presence of 0.05% SDS and 50 mM 2-mercaptoethanol and then incubated with or without 2 milliunits of endoglycosidase H (Boehringer Mannheim) in a buffer containing 70 mM sodium acetate, pH 5.2, and 0.8% Triton X-100 at 30 °C overnight (14). For digestion with peptide-N-glycosidase F, crude membrane protein, 1.6 μg was dena- tured in the presence of 0.05% SDS and 50 mM 2-mercaptoethanol and then incubated with or without 0.5 unit of PNGase F (Boehringer Mannheim) in a buffer containing 150 mM Tris-HCl, pH 8, and 1.3% Triton X-100 at 30 °C overnight (14).

Western Analysis of Plasma Membrane Proteins—After determination of the protein concentration in the crude plasma membrane preparations using the Pierce BCA protein assay (Pierce), an appropriate amount of membrane protein (4 μg) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (16) using a linear gradient of poly- acrylamide (4–12%). The proteins on the gel were subsequently electrically transferred to a nitrocellulose membrane. After blocking with 5% milk, the blot was incubated with the primary antibody (46411) used for immunocytochemistry with or without preincubation with the peptide (as a control) (17). After washing the blot (3×), the secondary, goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma; diluted 1:5000). The Ca2+-sensing re- ceptor protein was detected with an Enhanced Chemiluminescence system (Amersham Corp.).

Measurement of Ca2+ at Fluorimetry in Cell Populations—Coverslips coated with HEK293 cells that had been transfected with wild-type or mutant CaR cDNAs were loaded for 2 h at room temperature with fura-2/AM in 20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 1.25 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, 0.1% BSA, and 0.1% dextrose and washed once with a bath solution (20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl2, 0.5 mM MgCl2, 0.1% dextrose, and 0.1% BSA) at 37 °C for 20 min. The coverslips were then placed diagonally in a thermostatted quartz cuvette containing the bath solution, using a modified technique employed previously in this laboratory (17). CaR agonists were added to give desired concentrations. Excitation monochrometers were centered at 340 and 380 nm with emission light collected at 510 ± 40 nm through a wide band emission.
The $340/380$ excitation ratio of emitted light was used to calculate $\text{Ca}^{2+}$, as described previously (17). The addition of $\text{Ca}^{2+}$ with stepwise 1 mM increments was employed to characterize the wild type, the inactivating mutations (such as R62M and T138M, or the activating mutation (E127A). Increments of 5 mM were used for mutations with attenuated responses in order to study the effects of higher levels of $\text{Ca}^{2+}$.

Statistical Analysis—The mean EC$_{50}$ (an effective concentration of an agonist giving one-half of the maximal response) for the wild type or each mutant receptor in response to increasing concentrations of $\text{Ca}^{2+}$ or Gd$^{3+}$ was calculated from the EC$_{50}$ values for all of the individual experiments and is expressed with the standard error of the mean (S.E.) as the index of dispersion. Comparison of the EC$_{50}$ values was performed using analysis of variance (18) or Duncan’s multiple comparison test (19) ($p \leq 0.05$).

RESULTS

FHH/NSHPT and ADH Mutations Introduced into the Human CaR and the Restriction Map of the Reconstructed CaR Used for Site-directed Mutagenesis—Fig. 1 shows the activating and inactivating mutations engineered into the reconstructed human CaR as well as their positions relative to the predicted topology of the CaR. Fig. 2 shows the restriction maps for both the wild type and reconstructed human CaRs, illustrating the silent mutations introduced into the wild type CaR to create five unique restriction sites indicated by the arrows. Six mutagenesis cassettes shown below the map of rHuPCaR4.0 were constructed by flanking all pairs of unique restriction sites with XbaI (A) or KpnI (B) sites, which can be cloned into pBluescript SK$^+$. Details are given in under “Results” and “Experimental Procedures.”

Expression of the CaR in Transiently Transfected HEK293 Cells—We immunostained the HEK293 cells transiently transfected with rHuPCaR4.0 using pcDNA3 as a vector and LipofectAMINE as a carrier. The transfection efficiency of the receptor was estimated to be about 25%, as shown in Fig. 3. The immunostaining of the cells was specific, because it could be completely blocked by preincubation of the CaR antibody with the peptide against which it was raised (data not shown). Moreover, there was no staining of nontransfected HEK293 cells with the antiCaR antibody (data not shown).

Western Analysis of the CaR Expressed in HEK293 Cells—On Western analysis of crude plasma membrane proteins isolated from transiently transfected or wild type HEK293 cells using the same antiCaR antibody, there were three immunoreactive bands between 120 and 200 kDa as well as additional bands of...
higher molecular masses (~350 kDa) that were found only in the transfected cells and were ablated following preabsorption of the antibody with specific peptide (Fig. 4, the transfected cells and were ablated following preabsorption with higher molecular masses (195400.625 µg of the HuPCaR4.0 cDNA as described under “Experimental Procedures.” The transfected cells were then immunostained 48 h later using an anti-receptor antibody, 4641.

**Expression of Ca\(^{2+}\) Receptor Mutations**

**Fig. 3.** Immunocytochemical detection of the CaR protein on HEK293 cells transiently transfected with rHuPCaR4.0. The cells were plated on circular coverslips (within individual wells of 24-well plates) and transfected with 0.625 µg of the HuPCaR4.0 cDNA as described under “Experimental Procedures.” The transfected cells were then immunostained 48 h later using an anti-receptor antibody, 4641.

**Fig. 4.** Western analysis of CaR in a crude plasma membrane preparation isolated from HEK293 cells transiently transfected with rHuPCaR4.0. Crude plasma membrane proteins were isolated from HEK293 cells (grown in a 100-mm Petri dish) that had been transiently transfected with 15 µg of rHuPCaR4.0 cDNA or from a bovine parathyroid gland as described under “Experimental Procedures.” Each protein sample, 4 µg, was subjected to SDS-PAGE on a linear gradient running gel of 4–12%. The CaR was then stained with either an antireceptor antibody, 4641, in A or with the same antibody pretreated with the specific peptide against which it was raised as a control for nonspecific staining in B. In both A and B, lane 1 shows mock transfected HEK293 cells, lane 2 shows rHuPCaR4.0-transfected HEK293 cells, and lane 3 shows bovine parathyroid gland.

**Inhibition of Glycosylation of the CaR with Tunicamycin—**

The immunoreactive bands present as a doublet between 140 and 200 kDa in transfected HEK293 cells were absent in the cells treated with tunicamycin 48 h after transfection with rHuPCaR4.0 (Fig. 5). The band around 120 kDa in the crude plasma membranes isolated from the tunicamycin-treated cells co-migrated with the lowest band of the triplet between 120 and 200 kDa found in tunicamycin mock-treated cells in the same experiment.

**Characterization of Multiple Forms of the CaR Expressed in HEK293 Cells—**

The lower band of the doublet between 140 and 200 kDa was sensitive to endoglycosidase H as well as to PNGase F, but the upper band was only sensitive to PNGase F (Fig. 5). The species generated by endoglycosidase H co-migrated with the nonglycosylated form of the receptor at around 120 kDa (see tunicamycin-treated cells, Fig. 5). However, following treatment with PNGase F, the upper band migrated between 120 and 140 kDa in untreated cells. This suggests that the mature receptor may carry posttranslational modification(s) in addition to N-glycosylation.

**Western Analysis of Transfected CaRs Bearing FHH/NSSHPT and ADH Mutations—**

Crude plasma membranes were isolated from HEK293 cells transiently transfected with either rHuPCaR4.0 or receptors containing the mutations indicated in Fig. 1. On Western analysis the intensity of the upper band relative to the lower band of the doublet between 140 and 200 kDa was reduced in cells transfected with cDNAs carrying the R66C, G143E, and E297K mutations (Fig. 6). Nevertheless, some protein at the position of the upper band was detected for each of these three mutant receptors. The remaining mutant receptors exhibited an expression pattern on Western analysis similar to that of rHuPCaR4.0 (data not shown).

**Ca\(^{2+}\) Responses of rHuPCaR4.0 to Ca\(^{2+}\) or Which Are Similar to That of HuPCaR4.0—Reconstructed HuPCaR4.0 (rHuP-
Expression of Ca\(^{2+}\) Receptor Mutations

**Fig. 5. Use of enzymatic deglycosylation to analyze the carbohydrate content of rHuPCaR4.0 transiently expressed in HEK293 cells.** Crude plasma membrane proteins isolated from HEK293 cells (grown in a 100-mm Petri dish) transiently transfected with 15 μg of the rHuPCaR4.0 cDNA (Control) were treated (+) or mock-treated (−) with endoglycosidase H (Endo H) or PNGase F. Crude plasma membrane proteins (Tunicamycin) were also isolated from HEK293 cells that had been transiently transfected with rHuPCaR4.0 cDNA in the presence of tunicamycin as described under “Experimental Procedures.” Each sample, 1.6 μg of protein, was subjected to SDS-PAGE on a linear gradient running gel of 4–12%. The CaR proteins were stained with an antireceptor antibody, 4641, as described in the legend to Fig. 4.

![Fig. 5](image)

**Fig. 6. Western analysis of wild type and mutant receptors.** Crude plasma membrane proteins were isolated from HEK293 cells (grown in 100-mm Petri dishes) that were transiently transfected with 15 μg of the wild type rHuPCaR4.0 or with cDNAs containing each of the inactivating mutations shown in Fig. 1. The protein samples, 4 μg each, were subjected to SDS-PAGE on a linear gradient running gel of 4–12% (from left to right); mock-transfected (mock), wild type, R62M, R66C, T138M, G143E, R185Q, and R795W. The CaR proteins were stained with antireceptor antibody, 4641, as described in legends to the previous figures. The blot shown is representative of the pattern seen in three runs of each of two protein preparations from two independent transfections.

The amount of DNA that was used in subsequent transfections with a single mutant receptor cDNA to characterize seven previously identified FHH/NSHPT mutants was 2.5 μg, whereas 0.625 μg each of DNA was used in cotransfections of the wild type receptor cDNA with any one of the mutant receptors.

**Ca\(^{2+}\) Responses of Receptors Bearing FHH and NSHPT Mutations to Ca\(^{2+}\).** Among the seven inactivating mutations, R62M and T138M exhibited maximal responses to Ca\(^{2+}\) that were comparable with that of rHuPCaR4.0, but their dose response curves to Ca\(^{2+}\) were right-shifted with an EC\(_{50}\) of 5.6 ± 0.2 and 12.4 ± 0.4 mM, respectively (Fig. 7A and Table I). R66C, R185Q, and R795W exhibited substantially attenuated maximal responses with EC\(_{50}\) values of 15.8 ± 1.0, 28.4 ± 0.3, and 12.8 ± 0.3 mM, respectively (Fig. 7A and Table I). G143E and E297K did not respond to very high concentrations of Ca\(^{2+}\) (>50 mM). Mean Hill coefficients for the high Ca\(^{2+}\)-evoked increases in Ca\(^{2+}\) for the wild type and mutant receptors were close to 3.

**Ca\(^{2+}\) Responses of Receptors Bearing FHH and NSHPT Mutations to Gd\(^{3+}\).** Among the three mutant receptors, R62M and T138M had almost identical EC\(_{50}\) values of 15 ± 2 and 25 ± 3 μM, respectively, which were slightly higher than that of rHuPCaR4.0, 16 ± 2 μM. R185Q had a much higher EC\(_{50}\) of 145 ± 24 μM, whereas R66C, G143E, and E297K had attenuated maximal responses to Gd\(^{3+}\), as well as increased values for their EC\(_{50}\) values of 188 ± 19, 148 ± 14, and 131 ± 10 μM, respectively. R795W did not respond at all to extremely high concentrations of Gd\(^{3+}\) (>500 μM). Mean Hill coefficients for the Gd\(^{3+}\)-evoked increases in Ca\(^{2+}\) for the wild type receptor and mutant receptors were close to 1.

**Ca\(^{2+}\) Responses of Receptor Bearing an ADH Mutation to Ca\(^{2+}\).** A receptor bearing the mutation E127A exhibited a maximal response to Ca\(^{2+}\) that was similar to that of rHuPCaR4.0 but had a left-shifted dose response curve, with an EC\(_{50}\) of 3.3 ± 0.1 mM (n = 11) (Fig. 8).

**Discussion**

Multiple forms of the CaR were detected by Western blotting of crude plasma membrane proteins prepared from HEK293 cells transiently transfected with the receptor that were similar to those seen in membranes prepared from bovine parathyroid gland. Three bands between 120 and 200 kDa correspond to three distinct forms of the monomeric receptor varying in their states of glycosylation. The lowest band, around 120 kDa, is a nonglycosylated form which co-migrated with the band isolated from tunicamycin-treated cells. The middle band is a high mannose form of the receptor that was sensitive to endoglycosidase H as well as to peptide-N-glycosidase F. The uppermost of the three bands most likely represents the fully glycosylated, mature form of the receptor, which was only sensitive to peptide-N-glycosidase F. In addition to the monomeric forms, high molecular mass species (around 350 kDa) were observed as well, with varied sensitivities to endoglycosidases. The nature of these high molecular mass species is not clear. They may represent an oligomeric form of the receptor that is present in the transfected cells and is of some physiological relevance or might simply be nonspecific aggregates that are artifacts of protein extraction and preparation for electrophoresis.

Western analysis of expressed receptors containing FHH/NSHPT mutations provides some indirect evidence that the mature, glycosylated form of the receptor is important for full biological activity. For example, R66C, G143E, and E297K each had a substantially reduced amount of the mature, glycosylated form relative to the high mannose forms in comparison with those of the wild type receptor and the remaining mutants. Correspondingly, the maximal Ca\(^{2+}\) responses to CaR...
agonists for these three mutants was severely attenuated. The reduced amount of the mutant receptors containing higher molecular weight, complex carbohydrates may have resulted from unsuccessful cellular translocation of the mutant receptor from endoplasmic reticulum to Golgi where the high mannose forms of glycoproteins including receptors are thought to be processed to more mature forms. In contrast, receptors containing the mutations R62M, E127A, T138M, and R795W all showed expression patterns on Western blotting similar to that of the wild type receptor. Among these, R62M, E127A, T138M, and R185Q had maximal responses to Ca\(^{2+}\) or Gd\(^{3+}\) or both that were similar to those of the wild type receptor. All inactivating mutations had varying shifts to the right in their dose response curves to agonists. In contrast, the activating mutation, E127A, caused an apparent increase in the affinity of the receptor for Ca\(^{2+}\) with a shift to the left in its dose response curve.

These observations suggest that the mutations studied here within the extracellular domain, which reside exclusively within its first, amino-terminal half, modulate the affinity of the CaR for Ca\(^{2+}\) and other polycationic agonists without markedly affecting the capacity of the receptor to couple to intracellular signal transduction systems (in this instance phospholipase C). It is of interest in this regard that Hammerland et al. (20) have recently shown that chimeric receptors in FHH/NSHPT and ADH affect binding of the mutations R62M, E127A, T138M, R185Q, and R795W processed to more mature forms. In contrast, receptors containing the wild type receptor reach their maximum even at 50 mM Ca\(^{2+}\), which may indicate that the primary abnormality in receptor function is in ligand binding rather than signal transduction. This notion is supported by the nearly normal maximal response to Gd\(^{3+}\) another CaR agonist.

Interestingly, mutant receptors, like those containing R66C, G143E, or E297K that had little or no response to Ca\(^{2+}\) had some responses to Gd\(^{3+}\) which are proportional to the amounts of their mature, fully glycosylated forms. This observation raises the possibility that the Ca\(^{2+}\)-sensing receptor may have low affinity sites for interaction with Gd\(^{3+}\) that differ from those for Ca\(^{2+}\) or that disruption of the binding of Ca\(^{2+}\) to a common binding site(s) for both ions does not necessarily interfere as much with the binding affinity for Gd\(^{3+}\). Because the Ca\(^{2+}\) dose response curves for all of the FHH/NSHPT mutants were right-shifted in proportion to the right-shift in their Ca\(^{2+}\) dose response curves, this would support the idea that Ca\(^{2+}\) and Gd\(^{3+}\) may share the same binding site(s). However, R62M and T138M had very similar EC\(_{50}\) values for Gd\(^{3+}\), which were only slightly higher than that of the wild type receptor, despite the 2-3-fold difference in the EC\(_{50}\) values of R62M and T138M for Ca\(^{2+}\). The latter observation may favor the hypothesis that discrete but interrelated sites exist in the receptor for the two ligands. Indeed, more direct support for this hypothesis has come from the preliminary studies of Hammerland et al. (20). These workers produced a truncated form of the CaR lacking essentially all of its amino-terminal extracellular domain. Although this receptor failed to respond to high levels of Ca\(^{2+}\), it still showed a reasonably robust response to Gd\(^{3+}\) consistent with the presence of a binding site for Gd\(^{3+}\) in some part of the receptor other than its extracellular amino terminus. Although the number of binding sites both within and outside of the amino

### Table I

| Mutation | Single transfection* | Cotransfection | Elevation of serum calcium in FHH patients [\(\mu\text{M Ca}^{2+}\)] |
|----------|----------------------|----------------|--------------------------------------------------|
|          | EC\(_{50}\) [Ca\(^{2+}\)] | EC\(_{50}\) [Gd\(^{3+}\)] |                                                   |
|          | nm      | µM     | nm      | µM     | nm      | µM     |
| Wild type| 4.1 ± 0.1 (12)* | 16 ± 2 (10) | 4.1 ± 0.1 (17) | 0.30 ± 0.07 (7)* |
| R62M     | 5.6 ± 0.2 (6)*   | 25 ± 2 (7)*  | 4.3 ± 0.24 (4) | 0.31 ± 0.01 (4)* |
| R66C     | 15.8 ± 1.0 (4)*  | 188 ± 20 (4)* | 3.8 ± 0.1 (4)*  | 0.44 ± 0.02 (8)* |
| T138M    | 12.4 ± 0.4 (6)*  | 25 ± 3 (8)*   | 5.0 ± 0.2 (12)* | 0.32 ± 0.04 (6)* |
| G143E    | NR*       | 148 ± 14 (4)* | 4.4 ± 0.1 (4)*  | 0.76 ± 0.04 (9)* |
| R185Q    | 28.4 ± 0.3 (4)* | 145 ± 24 (3)* | 6.6 ± 0.4 (12)* | 0.42 ± 0.07 (3)* |
| E297K    | NR        | 131 ± 10 (4)* | 4.2 ± 0.1 (4)*  | 0.59 ± 0.06 (4)* |
| R795W    | 12.8 ± 0.3 (4)* | NR            |                   |                   |

* Concentrations of ionized extracellular Ca\(^{2+}\) or Gd\(^{3+}\). In these experiments, each mutant was cotransfected with the wild type receptor (EC\(_{50}\) [Ca\(^{2+}\)]).

The mean difference in total serum [Ca\(^{2+}\)] between each affected member and the mean value of unaffected members (n = 3-14 of the same family).

Values are means ± S.E.; values marked with asterisks are significantly (p ≤ 0.05) different from the wild type. The number of the experiments is indicated in the parentheses.

NR, no response.
terminus of the CaR cannot be directly inferred from our data, containing 0.1% BSA, whereas the responses to Gd3+ cooperative sites, whereas a Hill coefficient for the Ca2+ response of the wild type CaR to Gd 3+ indicates with a vertical bar through each point. The EC50 for each curve is presented as mean ± S.E. The means with different superscripts are significantly (p ≤ 0.05) different. In this experiment, 0.625 μg of each cDNA was employed to transiently transfected HEK293 cells plated on a rectangular coverslip (within individual wells of 12-well plates).

Fig. 7. High Ca2+-evoked increases in Ca2+ in fura-2-loaded HEK293 cells transiently transfected with wild type (rHuPCaR4.0) Ca2+-sensing receptor cDNA or with cDNAs containing the inactivating mutations shown in Fig. 1. Studies on the Ca2+ responses to elevated Ca2+ were carried out in a solution containing 0.1% BSA, whereas the responses to Gd3+ were performed in a solution without BSA as described under “Experimental Procedures.” All responses are normalized to the maximum response of the wild type receptor (rHuPCaR4.0). Each data point is the mean value of 3-12 measurements (see Table I). The standard error of the mean (S.E.) is indicated with a vertical bar through each point. Some error bars are smaller than the symbol. In this experiment, 2.5 μg of each cDNA was used to transfect HEK293 cells plated on a rectangular coverslip (in an individual well of 12-well plates).

centration, ranging from 0.3 to 0.76 mM (Table I) higher than the normal level of 2.40 ± 0.02 mM (n = 43). The extent of the elevation in serum calcium concentration in vivo is least (≤0.42 mM) for those mutant receptors with relatively low levels of the mature, glycosylated form of the CaR protein in vitro. Because these mutant receptors did not respond to Ca2+ or may not bind Ca2+ at all, they might not compete with the wild type receptor for G proteins and other elements of the signaling systems activated by the CaR (i.e. phospholipase C). In these cases, the mild elevation of serum calcium may simply result from the decrease in the dose of the normal CaR gene. It is of note that mice heterozygous for targeted disruption of the CaR gene also show a modest increase in serum calcium concentration (26) as do affected members of a FHH family with a mutation that introduces a premature stop codon in the extracellular domain of the CaR just proximal to the first membrane spanning segment (22). Therefore, in both of the two latter cases, the complete lack of functional receptor contributed by one allele is associated with a mild phenotype, supporting a role for gene dosage in determining the level of the CaR on the parathyroid cell surface in vivo. The very small or absent shift in most of the dose response to Ca2+ in our coexpression studies is most likely due to the fact that the number of CaRs on the cell surface of the HEK293 cells is not a limiting factor for activation of phospholipase C.

FHH patients harboring the mutations with patterns for protein expression similar to that of the wild type receptor (such as R62M, T138M, and R185Q but with the exception of R795W), on the other hand, have elevations of serum calcium that vary in proportion to their EC50 values when expressed transiently in HEK293 cells (see Table I and Fig. 7A). Among these, R185Q showed a prominent “dominant negative” effect on the coexpressed wild type receptor, which may contribute to the unusual elevation in the serum calcium concentration in
Fig. 9. Effect of coexpression of CaR cDNA containing the inactivating mutation, R185Q, with wild type receptor (rHuPCaR4.0) cDNA on Ca\(^{2+}\) evoked increases in Ca\(^{2+}\), in fura-2-loaded HEK293 cells. The Ca\(^{2+}\)-evoked increases in Ca\(^{2+}\), are reflected by the changes in the emission ratio (340/380 excitation), which is directly related to concomitant changes in Ca\(^{2+}\). At each arrowhead, the concentration of Ca\(^{2+}\) was increased to the indicated millimolar values. A: The Ca\(^{2+}\)-evoked increases in Ca\(^{2+}\) are reflected by the changes in the emission ratio (340/380 excitation), which is directly related to concomitant changes in Ca\(^{2+}\). At each arrowhead, the concentration of Ca\(^{2+}\) was increased to the indicated millimolar values. B: Cotransfection of a CaR cDNA containing the inactivating mutation, T138M, with wild type receptor. The mean EC\(_{50}\) for the activation of intracellular signaling pathways by a variety of hormones is usually severalfold to manyfold higher than that for the regulation of the biological response of a given tissue (27). Finally, further characterization and classification of FHH and ADH mutations may provide useful insights into the structure-function relationships of CaR.

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