Functional Characterization of Calcium-sensing Receptor Mutations Expressed in Human Embryonic Kidney Cells

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Abstract

The calcium-sensing receptor (CaR) is a G-protein-coupled receptor that plays a key role in extracellular calcium ion homeostasis. We have engineered 11 CaR mutants that have been described in the disorders familial benign hypercalcemia (FBH), neonatal severe hyperparathyroidism (NSHPT), and autosomal dominant hypocalcaemia (ADH), and studied their function by characterizing intracellular calcium $[Ca^{2+}]_i$ transients in response to varying concentrations of extracellular calcium $[Ca^{2+}]_o$ or gadolinium $[Gd^{3+}]_o$. The wild type receptor had an EC_{50} for calcium ($EC_{50}[Ca^{2+}]_o$) (the value of $[Ca^{2+}]_o$ producing half of the maximal increase in $[Ca^{2+}]_i$) of 4.0 mM (±0.1 SEM). However, five missense mutations associated with FBH or NSHPT, (P55L, N178D, P221S, R227L, and V817I) had significantly higher $EC_{50}[Ca^{2+}]_o$s of between 5.5 and 9.3 mM (all $P < 0.01$). Another FBH mutation, Y218S, had an $EC_{50}[Ca^{2+}]_o$ of > 50 mM but had only a mildly attenuated response to gadolinium, while the FBH mutations, R680C and P747fs, were unresponsive to either calcium or gadolinium. In contrast, three mutations associated with ADH, (F128L, T151M, and E191K), showed significantly reduced $EC_{50}[Ca^{2+}]_o$s of between 2.2 and 2.8 mM (all $P < 0.01$). These findings provide insights into the functional domains of the CaR and demonstrate that mutations which enhance or reduce the responsiveness of the CaR to $[Ca^{2+}]_o$ cause the disorders ADH, FBH, and NSHPT, respectively. (*J. Clin. Invest.* 1996. 98:1860–1866.) Key words: G protein-coupled receptor • hypercalcemia • neonatal hyperparathyroidism • hypocalcemia

Introduction

The cell surface calcium-sensing receptor (CaR)[1] is expressed in the parathyroid glands and kidneys where it allows regulation of parathyroid hormone (PTH) secretion and renal tubular calcium reabsorption appropriate to the prevailing extracellular calcium concentration $[Ca^{2+}]_o$ (1–5). The CaR is a member of the superfamily of G-protein–coupled receptors (GPCR) and has a large, 612 amino acid extracellular domain that is thought to bind calcium or other agonists such as magnesium, gadolinium, and neomycin (2–7). Ligand binding results in G-protein dependent stimulation of phospholipase C activity causing accumulation of inositol 1,4,5-trisphosphate (IP_3) (2, 8) and rapid release of calcium ions $[Ca^{2+}]_i$ from intracellular stores followed by an influx of extracellular calcium (9–11). These intracellular events are thought to mediate a decrease in the rate of PTH secretion from the parathyroid cell and a reduction in renal tubular calcium reabsorption (1–4). Thus, the CaR plays a key role in the regulation of extracellular calcium homeostasis. Structurally the CaR is an unusual member of the GPCR superfamily, in that it has the 7 hydrophobic transmembrane domains typical of such receptors but has little sequence homology to most other family members (2–5). Although members of the metabotropic glutamate receptor subfamily (mGluRs) do share limited homology to the CaR, this structural similarity is weak with only 30% amino acid identity between the CaR and the most homologous member, mGluR1 (2–5, 12). Thus, little is known about the structure–function relationships of this important receptor.

Mutations in GPCRs may cause either a loss of receptor function, as found in the type 2 vasopressin and the adrenocorticotropin receptors in the disorders of X-linked nephrogenic diabetes insipidus and familial glucocorticoid deficiency, respectively (13, 14), or a gain of receptor function, as found in the thyrotropin and luteinizing hormone receptors in the disorders of toxic thyroid adenoma and familial male precocious puberty, respectively (15, 16). Similar mutations in the CaR have been reported in association with two hypercalcaemic disorders, familial benign (hypocalciuric) hypercalcemia (FBH) and neonatal severe hyperparathyroidism (NSHPT) (17–22), and also in association with an autosomal dominant form of hypocalcemia (ADH) (23–25). Functional expression of five missense mutations associated with FBH in *Xenopus laevis* oocytes has shown that they cause a reduction in the $[Ca^{2+}]_o$-activated chloride influx that results from CaR-mediated activation of PLC compared to the wild type receptor, consistent with the predicted loss or reduction of CaR function (17, 20). Similarly, expression of a mutation found in a family with ADH in the *Xenopus* system confirmed an increase in basal and stimulated intracellular IP_3 accumulation, consistent with a gain of receptor function, although no difference in $[Ca^{2+}]_o$-activated chloride currents could be demonstrated (23). How-

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1. *Abbreviations used in this paper:* ADH, autosomal dominant hypocalcemia; CaR, calcium-sensing receptor; FBH, familial benign (hypocalciuric) hypercalcemia; GPCR, G-protein coupled receptors; IP_3, inositol 1,4,5-trisphosphate; NSHPT, neonatal severe hyperparathyroidism; pBSK, pBluescript; PTH, parathyroid hormone; WT, wild type.

ever, there are important differences between the CaR mediated responses observed in *X. laevis* oocytes compared to mammalian cells, as agonist dependent IP_3 accumulation is inhibited by pertussis toxin in the *Xenopus* system, whereas in dispersed mammalian parathyroid cells this response is pertussis toxin insensitive, suggesting differences in the specificity of the G protein coupling (1, 2). In order to gain further insight into the structure-function relationships of the CaR, we have expressed a further 11 mutations in the CaR that we have previously documented to be associated with FBH, NSHPT, or ADH (19, 25, 26) using a mammalian system and have characterized their $[Ca^{2+}]_i$ responses to varying concentrations of extracellular calcium or the more potent agonist gadolinium.

Methods

Site-directed mutagenesis. The wild type human CaR cDNA (HuPCaR4.0) (3) was ligated into pBluescript SK(−) (pBSK) (Stratagene, La Jolla, CA), and five unique silent restriction enzyme sites were introduced using oligonucleotides to give six cassettes, which ranged between 0.5 and 1kb in length, encompassing the entire CaR coding sequence. These cassettes were used to make single stranded DNA templates from pBSK(−) using helper phage for in vitro replication, and mutagenic oligonucleotides corresponding to the previously reported mutations (19, 25, 26) were annealed to these templates, ligated and used to make double stranded mutant DNA with T4 DNA polymerase (New England Biolabs, Beverly, MA) (27). The synthesized dsDNA mutant cassettes were used to transform DH5α cells (GIBCO-BRL, Gaithersburg, MD). Colonies containing the mutant construct were then screened for by PCR using pBSK(−) vector primers followed by restriction enzyme digestion, employing enzymes corresponding to the desired mutation (19, 25, 26). For mutations that did not alter a restriction site, a second silent base change was made in the mutagenic oligonucleotide to enable such screening. Mutant clones were sequenced in both directions using fluorochrome-labeled dideoxy terminators (Applied Biosystems, Foster City, CA). The mutated cassettes were then excised from the pBSK(−) and ligated back into the wild type CaR cDNA in a mammalian expression vector with a CMV promoter and bovine growth hormone 3′ untranslated region (pcDNA3; Invitrogen, San Diego, CA).

Cell culture and transfection. Human embryonic kidney cells (HEK 293, ATCC No. CRL-1573) were grown to 80-90% confluence on 13.5 × 20.1 mm glass cover slips in Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT). Transfection was performed using 2.5 μg of wild type or mutant DNA per cover slip which was first dissolved in 125 μl of serum-free medium (Opti-MEM; GIBCO-BRL) and then mixed with a suspension of 5% lipofectamine (GIBCO-BRL) in 250 μl of serum free medium. After incubation for 30 min at 20°C to allow DNA-liposome complexes to form, the cell-coated cover slip was immersed in a 1:5 dilution of this mixture with fresh serum-free medium and incubated at 37°C for 5 h. This was followed by addition of an equal volume of serum-free medium supplemented with 20% fetal calf serum. Co-transfection of two clones was carried out in a similar manner except that only 0.5 μg of each DNA were dissolved in the 125 μl of serum-free medium before addition of the liposome solution. Functional characterization of CaR activity in the cells was performed 36–48 h after transfection.

Determination of $[Ca^{2+}]_o$- and $[Gd^{3+}]_o$-induced changes in $[Ca^{2+}]_i$. The fluorescent probe fura-2 was used to monitor the $[Ca^{2+}]_i$ response of transfected HEK-293 cells upon exposure to varying levels of $[Ca^{2+}]_o$ or $[Gd^{3+}]_o$ (11, 28), and each mutant construct was studied on at least two occasions with separate transfections. Cover slips were loaded by incubation of transfected cells with 4 μM of the acetoxymethyl ester of fura-2 (Molecular Probes, Eugene, OR) for 2 h at room temperature in 1 ml of a buffer containing 130 mM NaCl, 4 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM Hepes, 1% dextrose, and 1% BSA (pH 7.4 at 37°C), which was used for all further manipulations. After this the cover slips were washed for 20 min before insertion into a quartz cuvette fitted with a magnetic stir bar in a thermostatted platform maintained at 37°C. A dual wavelength xenon light source was used with a pair of monochrometers centered at 340 and 380 nm to excite the fura-2, and the emitted signal, filtered at 510±40 nm was detected with a photon-counting photomultiplier tube (Photon Technology International, Princeton, NJ), as described (29). To assess $[Ca^{2+}]_o$- or $[Gd^{3+}]_o$-evoked changes in $[Ca^{2+}]_i$ the magnitude of the peak of the transient response after an individual stimulus (δ_n) was added to the sum of the counts of the previous stimuli and expressed as a proportion of the total counts for the full concentration-response curve up to the maximal response (Σ^{δ}_{n}) (at which no further $[Ca^{2+}]_i$ increment occurred), to produce a "normalized" response (R_n). Thus, $R_n = \delta_x+\delta_{x-1}+\delta_{x-2} \ldots /\sigma\delta_n$ and for example, for the response to 4.5 mM $[Ca^{2+}]_o$ (δ_3) (Fig. 1), the normalized $[Ca^{2+}]_i$ response would be $\{\delta_1+\delta_2+\delta_3/\Sigma\delta_n\}$. The magnitude of the maximal response ($\Sigma\delta_n$) varied depending on the cell density and the level of fura-2 loading, but there was a consistent relationship between the responses of each mutant and that of the wild type examined on the same day. Moreover, this cumulative peak concentration-response curve was similar to that determined by combining several experiments where $[Ca^{2+}]_o$ was increased from 0.5 mM to a single higher level (e.g., 2.5 or 5.0 mM). Individual EC_{50}s for each incremental, normalized concentration–response curve were plotted, and the mean EC_{50} for several experiments was used for statistical comparisons.

HEK cell membrane preparation and Western analysis. Membrane fractions were isolated from confluent cultures of HEK-293 cells that had been transfected with 15 μg of wild type or mutant CaR constructs 48 h previously. Cells were dislodged with 0.02% EDTA in PBS, pelleted, and resuspended in 300 μl of homogenization buffer (50 mM Tris-HCl, pH7.4; 0.32 M sucrose; 1 mM EDTA; aprotinin 83 μg/ml; leupeptin 30 μg/ml; pefabloc 1 mg/ml; calpain inhibitor 50 μg/ml; bestatin 50 μg/ml; pepstatin 5 μg/ml) at 4°C. The cells were then homogenized with 15 strokes of a teflon pestle and nuclei and mitochondria sedimented at 18,800 *g* for 20 min, followed by 43,500 *g* for 20 min to pellet the membranes. Membrane pellets were solubilized in 1% Triton X-100 and 8 μg of protein was separated by SDS-polyacrylamide gel electrophoresis on a linear gradient (4–12%). The proteins on the gel were electrotransferred to nitrocellulose membranes and after blocking with 5% milk, the blots were incubated with 1:3000 dilution of antiserum (4641) raised against a synthetic peptide corresponding to residues 215–237 of the CaR. The 4641 antiserum has been previously demonstrated to be CaR specific, with binding abolished by peptide pre-incubation, and to recognize identical bands in preparations of bovine parathyroid cell membranes as in CaR transfected HEK-293 cell membranes, with no immunostaining of untransfected HEK cells (30). A goat anti-rabbit secondary antibody (Sigma Chemical Co., St. Louis, MO) was used which was conjugated to horseradish peroxidase enabling detection of the CaR protein by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Statistical analysis. The mean EC_{50} for each clone in response to $[Ca^{2+}]_o$ or $[Gd^{3+}]_o$ was calculated from the EC_{50}s for all of the individual incremental experiments and was expressed with the SEM. Comparison of the EC_{50}s was performed using analysis of variance (ANOVA) or Duncan's multiple range test (31).

Results

Assessment of $[Ca^{2+}]_i$ response of the wild type CaR to $[Ca^{2+}]_o$ and $[Gd^{3+}]_o$. We initially tested a series of incremental concentration regimes to assess the range of $[Ca^{2+}]_o$ and $[Gd^{3+}]_o$ over which $[Ca^{2+}]_i$ responses were observed for the wild type (WT) receptor and for the various loss- and gain-of-function mutants (Fig. 2). Preliminary experiments revealed that HEK cells transfected with WT CaR cDNA had a threshold for a re-

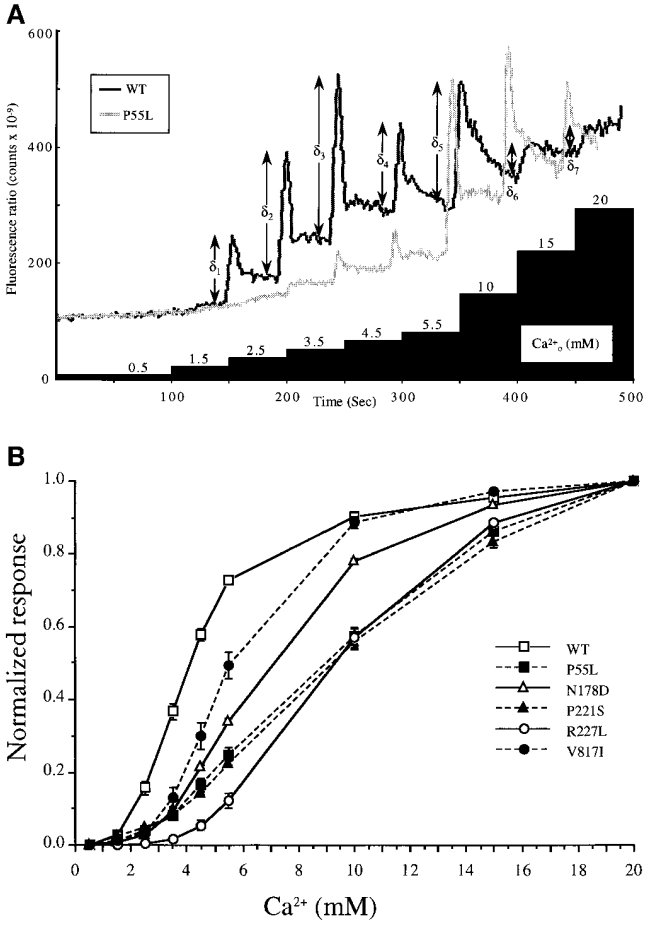

Figure 1. Fluorescence responses of CaR-transfected HEK cells to increasing levels of $[Ca^{2+}]_o$ (1.0 mM ladder-see results) (*A*) and the responses of hypercalcaemic mutations (*B*). *A* shows fluorescence responses observed in 2 typical experiments using WT receptor (*black*) or the mutant receptor, P55L (*gray*). For each increment in $[Ca^{2+}]_o$ there is a discrete peak in the ratio of the emmited fluorescence counts (at 340 vs 380 nm excitation) reflecting concomitant changes in $[Ca^{2+}]_i$ (*y*-axis), the magnitude of which we have designated δ_n. It can be seen from these data that the threshold for a $[Ca^{2+}]_o$. response in cells expressing the WT receptor is around 2.5 mM compared to 3.5 mM for P55L. It can also be seen that the concentration–response curve of the mutant receptor is shifted rightwards compared to the WT CaR. The method of normalization of the fluorescence ratio is described in detail in Methods. Hypercalcaemic mutations (*B*). Normalized response of transiently transfected HEK cells expressing the WT receptor or CaRs containing 4 FBH mutations (P55L, N178D, P221S, V817I) and an NSHPT mutation (R227L) to increasing levels of $[Ca^{2+}]_o$. The increments in $[Ca^{2+}]_o$ were 1 mM from the baseline of 0.5 mM up to 5.5 mM, after which $[Ca^{2+}]_o$ was increased to 10, 15, and 20 mM Ca^{2+}_o. Vertical bars through points represent the SEM for each point. The concentration-response curves are similar to the sigmoidal curve observed in vitro for $[Ca^{2+}]_o$-regulated PTH release in parathyroid cells, and the EC_{50}s of these mutations are all significantly different from that for the WT CaR (Table I).

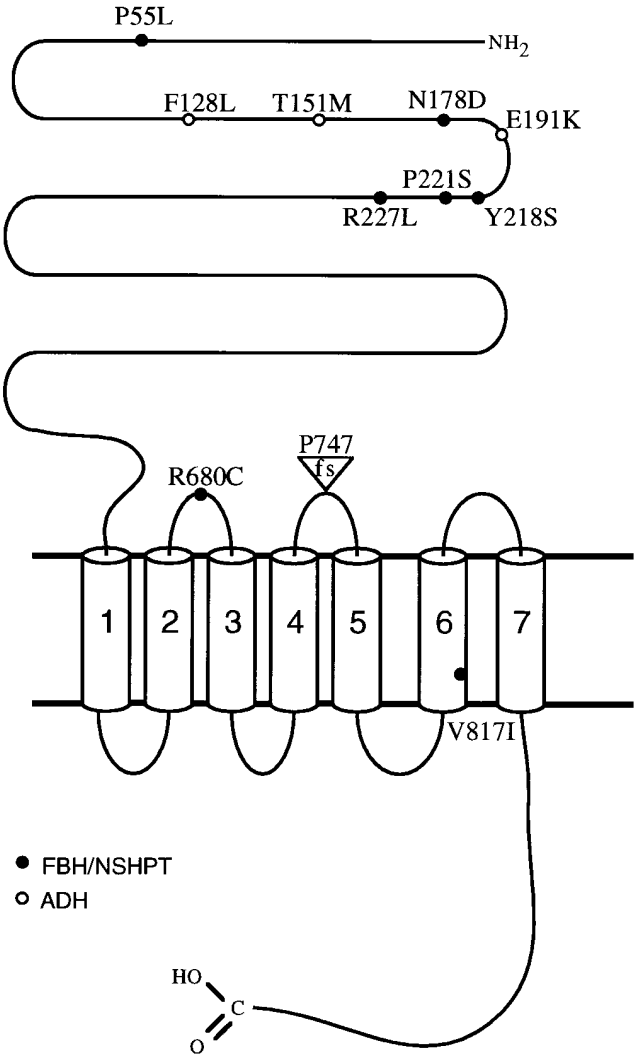

Figure 2. Topographical representation of the human CaR showing the position of the 11 CaR mutants that were engineered and expressed in human embryonic kidney cells. The triangular symbol (fs) represents the position of the 1bp deletion which results in the frameshift mutation, P747fs, and a truncated receptor lacking 3 transmembrane domains.

sponse to $[Ca^{2+}]_o$ of between 1.5 and 2.0 mM, an $EC_{50}[Ca^{2+}]_o$ of between 3.5 and 4.5 mM and a near maximal (95% or greater) response at 15 mM Ca^{2+}_o. A multiple-concentration regimen, using five 1 mM increments in $[Ca^{2+}]_o$ from the basal level of 0.5 mM to 5.5 mM, followed by further increments to levels of $[Ca^{2+}]_o$ of 10, 15, and 20 mM, was found to yield sharp transient peaks in $[Ca^{2+}]_i$ with a reproducible EC_{50} of 4.0 mM (±0.1, SEM) for the WT receptor (Fig. 1). A Hill plot of these concentration–response curves gave a mean Hill coefficient of 3.1±0.1. The sigmoidal nature of this concentration-response curve (Fig. 1) was consistent with that which we observed by combining several experiments in which a single concentration of $[Ca^{2+}]_o$ was added to each cover slip in this system, with the $EC_{50}[Ca^{2+}]_o$ differing by less than 10% between the multiple increment compared to the single stimulus experiments. Similarly, the concentration–response curve was also consistent with that previously observed for the native receptor both in vivo and in vitro in parathyroid cells and in vitro in *Xenopus laevis* oocytes injected with cRNA encoding the CaR (1–5, 9–11). Although this "1 mM" incremental $[Ca^{2+}]_o$ "ladder" allowed easy differentiation of the WT response from that of inactivating CaR mutations, it was not sufficiently sensitive to allow good characterization of the response to levels of $[Ca^{2+}]_o$

in the range of 1.5–3 mM, which coincided with the apparent $EC_{50}[Ca^{2+}]_o$s for the CaR mutations associated with autosomal dominant hypocalcemia. To this end, a second incremental $[Ca^{2+}]_o$ ladder was tested, using 0.5 mM $Ca^{2+}{}_o$ increments from the basal level of 0.5 mM to 4 mM followed by a single increment to a final concentration of 10 mM $Ca^{2+}{}_o$. This regimen gave an $EC_{50}[Ca^{2+}]_o$ of 3.7 mM (±0.05) for the WT receptor and allowed detailed characterization of the $[Ca^{2+}]_i$ response at low levels of $[Ca^{2+}]_o$. The response of the WT receptor and CaRs with loss-of-function mutations to $[Gd^{3+}]_o$ was assessed using an incremental concentration regimen of 20, 40, 100, 200 μM $Gd^{3+}{}_o$ from a basal value of 0 μM. The $EC_{50}[Gd^{3+}{}_o]$ of the wild type receptor was 47 μM (±4.3). However, the responses to $[Gd^{3+}]_o$ were always less consistent than those seen with $[Ca^{2+}]_o$, and no clone yielded sharp $[Ca^{2+}]_i$ transients to more than three concentration increments of $[Gd^{3+}]_o$.

Functional assessment of CaR mutations associated with FBH and NSHPT. Two of the eight mutations associated with FBH or NSHPT, R680C and P747fs (Fig. 2), showed no responses to either $[Ca^{2+}]_o$ at levels of up to 100 mM or to concentrations of $[Gd^{3+}]_o$ as high as 300 μM. A single FBH mutation, Y218S, had a blunted response to very high concentrations of $[Ca^{2+}]_o$ with an EC_{50} of over 50 mM on four of the seven occasions on which this was studied. This response to high $[Ca^{2+}]_o$ was not observed in sham-transfected HEK cells. This mutant, however, gave a brisk and only mildly attenuated response to $[Gd^{3+}]_o$ on all of four occasions with an $EC_{50}[Gd^{3+}]_o$ of 74 μM. The remaining five FBH and NSHPT mutations had elevated $EC_{50}[Ca^{2+}]_o$s ranging from 5.5 mM to 9.3 mM (Table I) with a rightward shift of the concentration–response curves to $[Ca^{2+}]_o$ as shown in Fig. 1. The Hill coefficients of the $Ca^{2+}{}_o$ concentration–response curves for these mutations all remained close to three and were not substantially different from that of the WT receptor. The maximal responses of these mutant clones when compared to the WT response assessed on the same day was consistently reduced or increased. For example, P55L gave a maximal response that was 81% (±2%) of WT on two different days. The mutations, N178D, P221S, R227L, and V817I, all gave similar maximal responses of 104%, 110%, 113%, and 94%, respectively, compared to WT. Rightward shifts in $EC_{50}[Gd_3+]_o$ were also observed for three of these 5 FBH and NSHPT mutations (Table I), although there was no clear correlation between the changes in EC_{50} observed for $[Ca^{2+}]_o$ and $[Gd^{3+}]_o$. To examine the possible role of gene dosage effects in vitro, we co-transfected the mutation R227L, which was found as a heterozygous mutation in a child with a form of NSHPT (19), with an equal amount of WT receptor cDNA. Thus, the HEK-293 cells were rendered "heterozygous" for this mutation, which created a large rightward shift in $EC_{50}[Ca^{2+}]_o$ when transfected alone ("homozygous") (Table I). The cells co-transfected with both WT and R227L DNA (0.5 μg each) had an $EC_{50}[Ca^{2+}]_o$ of 5.1 mM (±0.13), which differed significantly from both that for WT receptor DNA (0.5 μg) alone [4.3 mM (±0.08)] and for R227L (0.5 μg) alone [8.5 mM (±0.25)] ($P < 0.01$, Duncan test) (Fig. 3).

Functional assessment of CaR mutations associated with autosomal dominant hypocalcemia. Using a 0.5 mM incremental $[Ca^{2+}]_o$ concentration regimen, the mean EC_{50} of the WT receptor was 3.7 mM (±0.05). The mean $EC_{50}[Ca^{2+}]_o$s of three mutations (F128L, T151M, and E191K) associated with ADH were 2.2 mM (±0.05), 2.7 mM (±0.04), and 2.8 mM (±0.06) (all $n = 6$), respectively. These $EC_{50}[Ca^{2+}]_o$s all demonstrate a significant ($P < 0.01$) leftward shift compared to WT using the same 0.5 mM incremental ladder (Fig. 4). The maximal responses of each of these mutations were once again consistent when compared to WT controls assessed with the same incremental concentration ladder on the same day, such that the maximal responses were 94%, 130%, and 137% of the WT response for the mutants F128L, T151M, and E191K, respectively.

Table I. $EC_{50}[Ca^{2+}]_o$ FBH and $EC_{50}[Gd^{3+}]_o$ of Wild Type CaR, and Different FBHH and NSHPT Mutations

Clone	$EC_{50}[Ca^{2+}]_o$	SEM	*n*	$EC_{50}[Gd^{3+}]_o$	SEM	*n*
Wild type	4.0 mM	0.11	7	47 μM	4.3	4
P55L	8.9 mM*	0.28	5	74 μM*	1.5	3
N178D	7.0 mM*	0.07	6	68 μM*	1.7	5
Y218S	> 50 mM	—	4	74 μM*	1.5	4
P221S	9.2 mM*	0.24	5	78 μM*	6.4	3
R227L	9.3 mM*	0.26	6	48 μM	4.9	4
R680C	NR	—	4	NR	—	4
P747fs	NR	—	4	NR	—	4
V817I	5.5 mM*	0.25	6	42 μM	6.8	4

*$P < 0.01$ compared to WT. NR, no response to Ca^{2+} 100mM or Gd^{3+} 300μM.

Western analysis of expressed wild type and mutant CaR proteins. Membrane fractions from the HEK-293 cells that had been transfected with either WT or the five mutant CaR clones P55L, N178D, R227L, R680C, and V817I showed three immunoreactive bands between 120 and 160 kD (Fig. 5), which were consistent with the observed size of the CaR in parathyroid cell membranes (30) and which were abolished by peptide preabsorption. The frameshift mutation P747fs, which predicts

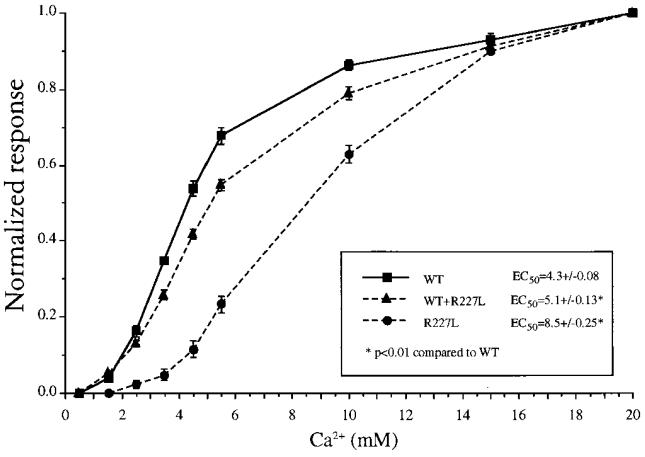

Figure 3. Cotransfection of WT CaR with the NSHPT mutation, R227L. 0.5 μg of WT CaR cDNA or 0.5 μg of R227L cDNA were transfected alone or 0.5 μg of both DNAs were mixed and cotransfected ($n = 4$ for each experiment). The EC_{50} for WT CaR in this experiment is slightly higher (4.3 mM±0.08) than that seen with transfection of 2.5 μg of WT DNA (4.0±0.11). The cotransfected HEK cells show an $EC_{50}[Ca^{2+}]_o$ that is right-shifted compared to that seen in cells transfected with WT alone ($P < 0.01$) and is also significantly different from that in cells transfected with only R227L DNA. Thus the presence of the mutant CaR causes a shift in the $EC_{50}[Ca^{2+}]_o$ to the right despite the presence of the normal CaR.

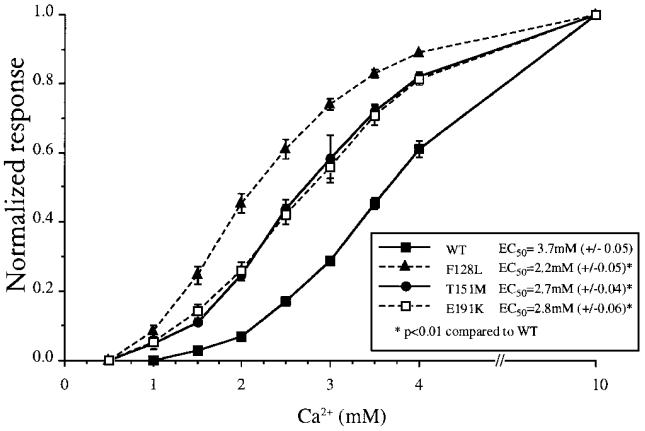

Figure 4. Hypocalcemic mutations. $[Ca^{2+}]_o$ was increased from a base-line value of 0.5 mM in 0.5 mM increments to 4.0 mM, then a single concentration of 10 mM was applied. The X-axis shows the increasing $[Ca^{2+}]_o$ concentrations up to this 10 mM maximum, in distinction from the 20mM maximum used in other series of experiments (Fig. 1 *b* and Fig. 3). Responses of $[Ca^{2+}]_i$ were measured and normalized for the maximal $[Ca^{2+}]_i$ response, as described in methods. The EC_{50} for the WT receptor was significantly different from that of each of the three mutant CaRs, which were all significantly left-shifted ($P < 0.01$). Vertical bars through points represent SEMs.

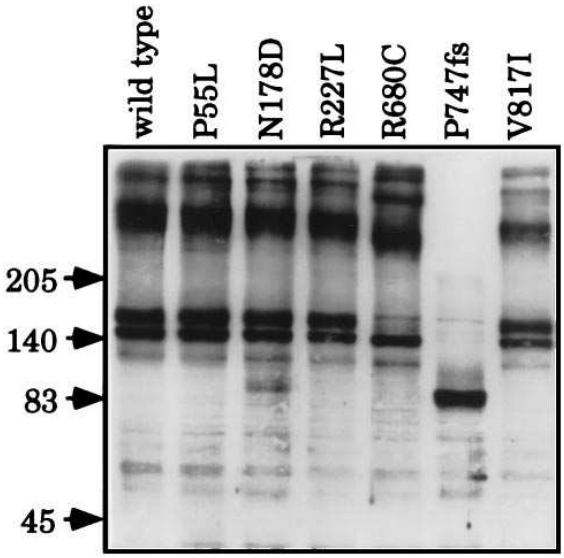

Figure 5. Western blot of crude cell membranes from transfected HEK-293 cells immunostained with the CaR antibody, 4641. The specificity of this antibody for the CaR and inhibition of its binding following preincubation with the synthetic peptide corresponding to residues 215–237 of the CaR has been reported previously (30). Cells transfected with the WT CaR cDNA can be seen to express three immunostainable bands between 120 and 160 kD. The cells transfected with the 4 loss of CaR function mutations, P55L, N178D, R227L, and V817I all demonstrate a similar quantity of immunoreactive receptor protein. The frameshift mutation, P747fs, which predicts a CaR lacking 3 transmembrane domains and the intracellular tail shows a smaller band of about 80 kD, whereas the mutation R680C has a reduced intensity of the 160-kD band compared to WT, suggesting reduced quantities of N-linked glycosylation or other post-translational modification of this mutant.

a CaR truncated at codon 775 (19) showed a smaller band of about 80 kD (Fig. 5) consistent with a truncated protein. The quantity of the expressed WT CaR protein was comparable to that of the six mutant CaRs (P55L, N178D, R227L, R680C, P747fs, V817I) (Fig. 5), thereby indicating that the abnormal functional responses of the mutant CaRs are not due to a lack of receptor expression on the cell membrane. However, as the 4641 antibody was raised against residues 215–237 of the CaR (30), we were unable to assess the expression of the 2 mutations, Y218S and P221S, which are predicted to interfere with the conformation of the antibody binding site.

Discussion

We have characterized the $[Ca^{2+}]_i$ responses of 11 CaR mutants (8 associated with loss of function and 3 associated with gain of function) (Fig. 2) expressed in human embryonic kidney cells to stepwise increases in $[Ca^{2+}]_o$. Our results show that three different classes of inactivating CaR mutants may exist. First, two of the mutations (R680C and P747fs) associated with FBH and NSHPT gave no response to either calcium or gadolinium. The P747fs mutation predicts a CaR which lacks three transmembrane domains. It would, therefore, probably be unable to couple normally to the associated G protein(s), which is thought to occur via the third intracellular loop in the majority of GPCRs (17, 31, 32) although in the structurally homologous metabotropic glutamate receptor subfamily, the second intracellular loop appears to be particularly important in this regard (34). Our finding that this receptor is expressed (Fig. 5) but is nonfunctional supports this hypothesis, although it is possible that that the abnormal receptor protein fails to reach the cell surface. The other nonfunctional mutation, R680C, results in a novel cysteine residue in the first extracellular loop. Many other GPCRs have a disulfide bridge between the first and second extracellular loops which has been shown to be important for maintenance of secondary structure (32, 33). It is possible that the novel cysteine of this mutation forms an aberrant bridge with one of the other cysteines and that this disrupts the critical relationship of the transmembrane domains, thereby preventing signal transduction. Alternatively, such an abnormal disulfide bridge might destabilize the protein and/or reduce the expression of its glycosylated forms on the cell surface (Fig. 5).

The second class of mutations are those that cause a rightward shift in the $EC_{50}[Ca^{2+}]_o$ of the CaR (Fig. 1). Four of these are in the extracellular domain of the receptor where they either predict structural change (i.e., loss of a "helix-breaking" proline, P55L and P221S) or net loss of positive charge (N178D and R227L). It is probable that these mutations alter the structure or charge distribution of the ligand-binding site(s) in such a way as to reduce the affinity of the receptor for cations. The remaining mutation of this class is the conservative V817I mutation which occurs within the hydrophobic sixth transmembrane domain of the CaR and produces a subtle shift in response ($EC_{50}[Ca^{2+}]_o$ = 5.5 mM). It seems possible that the additional methylene group of the isoleucine residue may stabilize this transmembrane domain and inhibit a putative conformational change necessary to activate the G protein(s). The third type of inactivating mutation that we have studied is the FBH mutation Y218S. This mutation produces a CaR with an attenuated and qualitatively different response to calcium

($EC_{50}[Ca^{2+}]_o > 50$ mM) but has only a modest rightward shift in EC_{50} for gadolinium ($EC_{50}[Gd^{3+}_o] = 74$ μM). This finding, taken together with the lack of a consistent relationship between the EC_{50}s for $[Ca^{2+}]_o$ and $[Gd^{3+}]_o$ in the mutations examined, raises the possibility that the CaR may have an additional "Gd^{3+}-binding" site(s) that interacts with Ca^{2+} only with lower affinity in this mutant receptor. The Hill coefficient of 3 that we have obtained for the WT receptor, the cooperativity between calcium and magnesium ions for rat CaR responses in vitro (35), and the observation that an amino-terminal CaR deletion mutant (ΔntCaR) that lacks most of the extracellular domain responds to $[Gd^{3+}]_o$ but poorly if at all to $[Ca^{2+}]_o$ (36) all support the idea that the CaR may have either different or overlapping ligand-binding sites for calcium and gadolinium.

As heterozygous CaR mutations are found in most subjects with FBH (17–22) and in some individuals with NSHPT (19), we co-transfected HEK cells with both WT and mutant (R227L) cDNAs to produce "heterozygosity" in vitro. The $EC_{50}[Ca^{2+}]_o$ of these "heterozygous" cells was right-shifted and approximately intermediate between that observed for the wild type receptor and for R227L cDNA when transfected alone (Fig. 3). Thus, the presence of the WT CaR is not able to "rescue" the EC_{50} of cells expressing this mutant CaR. Additional studies are necessary to determine whether the mutant receptor exerts a true dominant-negative effect, for instance by interfering with WT receptors by dimerization, or simply reduces the effective concentration of WT receptor on the cell surface, as found in mice heterozygous for a disruption of the CaR gene, which manifest mild hypercalcemia (37).

We have also demonstrated that three mutations associated with hypocalcemia appear to cause gain of receptor function by lowering the $EC_{50}[Ca^{2+}]_o$ for $[Ca^{2+}]_o$-elicited increase in $[Ca^{2+}]_i$ (Fig. 4). This is consistent with the levels of IP_3 documented for another such mutation when expressed in the *Xenopus* system (23). The left-shifted $EC_{50}[Ca^{2+}]_o$s of these mutations would explain their dominant nature in vivo, as PTH secretion is negatively regulated by CaR activation (1, 9) and it would be predicted, therefore, that inhibition of PTH secretion would occur at an inappropriately low concentration of $[Ca^{2+}]_o$ to produce a stable level of PTH secretion at a lower $[Ca^{+}]_o$. All of these mutations are within the extracellular domain of the receptor and two of them, F128L and T151M, predict a structural change, whereas the other, E191K, predicts the gain of a positive charge. It seems likely that these mutations alter the ligand-binding site(s) in such a way as to enhance the affinity of the receptor for $[Ca^{2+}]_o$, producing CaR activation at lower than normal levels of $[Ca^{2+}]_o$. Thus, we have demonstrated that these different CaR mutants may cause loss or gain of $[Ca^{2+}]_o$-responsive CaR function which is correlated clinically with the disorders FBH and NSHPT, and ADH, respectively. Furthermore, these studies have also provided insights into the possible nature and specificity of the CaR's ligand-binding site(s) (35), and of the predicted structure-function relationships (2–5) of this novel G-protein-coupled receptor.

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