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Loss-of-Function and Gain-of-Function Mutations of Calcium-Sensing Receptor: Functional Analysis and the Effect of Allosteric Modulators NPS R-568 and NPS 2143

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Objective: Activating mutations in the calcium-sensing receptor (CASR) gene cause autosomal dominant hypoparathyroidism, and heterozygous inactivating CASR mutations cause familial hypocalciuric hypercalcemia. Recently, there has been a focus on the use of allosteric modulators to restore the functional activity of mutant CASRs. In this study, the effect of allosteric modulators NPS R-568 and NPS 2143 on CASR mutants was studied in vitro.

Methods: DNA sequence analysis of the *CASR* gene was undertaken in autosomal dominant hypoparathyroidism and familial hypocalciuric hypercalcemia Japanese patients, and the functional consequences for the Gi-MAPK pathway and cell surface expression of CASR were determined. Furthermore, we studied the effect of NPS R-568 and NPS 2143 on the signal transduction activity and cell surface expression of each mutant CASR.

Results: We identified 3 activating mutations (S122C, P569H, and I839T) and 2 inactivating mutations (A110T and R172G) in patients. The activating and inactivating mutations caused leftward and rightward shifts, respectively, in the dose-response curves of the signaling pathway. NPS R-568 rescued the signal transduction capacity of 2 inactivating mutants without increasing cell surface expression levels. NPS 2143 suppressed the enhanced activity of the activating mutants without altering cell surface expression levels, although A843E, which is a constitutively active mutant, was suppressed to a lesser degree.

Conclusions: We have identified 4 novel mutations of *CASR*. Moreover, our results indicate that allosteric modulators can restore the activity of the loss- and gain-of-function mutant CASRs, identified in this study. (***J Clin Endocrinol Metab*** **98: E1692–E1701, 2013)**

Regulation of the extracellular ionized calcium concentration in a narrow range (1.1–1.3 mM) is affected in part by the calcium-sensing receptor (CASR). CASR is expressed in several organs related to calcium homeostasis, including the parathyroid gland, kidney, thyroid C cells, and small and large intestines. Activation of the CASR by increased extracellular calcium levels in the parathyroid gland inhibits PTH secretion (1–3), and activation of the CASR in the kidney inhibits renal tubular reabsorption of calcium and increases urinary calcium excretion (1–3).

CASR, which belongs to the family C group of G protein-coupled receptors, consists of 1078 amino acid resi-

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Abbreviations: ADH, autosomal dominant hypoparathyroidism; CASR, calcium-sensing receptor; ECD, extracellular domain; FACS, fluorescence-activated cell sorting; FHH, familial hypocalciuric hypercalcemia; HEK293, human embryonic kidney 293; TMD, transmembrane domain.

dues and has 3 major domains: 1) a large extracellular domain (ECD), which is comprised of a Venus flytrap domain and a cysteine-rich domain; 2) a transmembrane domain (TMD); and 3) an intracellular, cytosolic carboxy (C)-terminal domain (1, 4, 5). Stimulation of the CASR by extracellular calcium concentration can activate different transduction pathways, such as the Gq and Gi signaling pathways (2, 3, 5).

More than 250 mutations in the *CASR* have been identified (CaSR database: http://www.casrdb.mcgill.ca/). Activating mutations cause autosomal dominant hypoparathyroidism (ADH) (OMIM 146200), whereas inactivating mutations cause neonatal severe hyperparathyroidism (OMIM 239200) and familial hypocalciuric hypercalcemia (FHH) (OMIM 145980) (2–8). Activating mutations cause inhibition of PTH secretion and decreased renal calcium reabsorption, despite low serum calcium levels, leading to hypocalcemia and relative hypercalciuria in ADH patients. ADH patients are generally treated with calcium and active vitamin D, but these treatments sometimes result in nephrocalcinosis and renal dysfunction (5, 7, 8). FHH is caused by heterozygous inactivating mutations in *CASR*. FHH is characterized by moderately elevated serum calcium concentrations, increased renal calcium reabsorption, and normal or inappropriately elevated PTH levels (2–6). Most FHH patients are asymptomatic, are diagnosed by chance, and generally do not require medical treatment. By contrast, neonatal severe hyperparathyroidism, which is generally caused by homozygous inactivating mutations of *CASR*, is characterized by severe hypercalcemia just after birth and requires total parathyroidectomy (2–6).

Positive allosteric modulators (calcimimetics), including first-generation compounds such as NPS R-568, increase CASR activation in the presence of Ca^{2+}, and negative allosteric modulators (calcilytics), including NPS 2143, antagonize the stimulatory effects of Ca^{2+} (9–12). These allosteric modulators bind CASR within the TMD and either increase or decrease the apparent affinity of CASR for Ca^{2+} (10–12). In addition, it has been reported that calcimimetics such as NPS R-568 enhance the cell surface expression of several inactivating CASR mutants (13, 14). Cinacalcet is a first-in-class G protein-coupled receptor allosteric modulator approved by the US Food and Drug Administration for secondary hyperparathyroidism in patients receiving hemodialysis (15). In FHH, hypercalcemia is usually mild and most patients are asymptomatic, although in some cases, hypercalcemia is severe and is associated with significant complications that were difficult to control. Recently, calcimimetics have been shown to be useful for the treatment of such severe FHH patients (16–18). Moreover, it has been reported that calcilytics, such as NPS 2143 and AXT914, suppress the activity of gain-of-function mutants in vitro and calcilytics may be a treatment option for ADH patients (12, 19–21).

In this study, we identified mutations of *CASR* in ADH and FHH patients. Furthermore, the effects of NPS R-568 and NPS2143 on signal transduction and cell surface expression of the CASR mutants identified in this study were analyzed.

Subjects and Methods

Subjects

Patients were referred for endocrine evaluation of low or elevated serum calcium levels with inappropriately elevated or low urinary calcium excretion. Written informed consent to participate in the study was obtained from the patients and their parents. The ethical committee of Hokkaido University School of Medicine approved this study.

Sequence analysis of CASR gene

Genomic DNA was extracted from peripheral blood leukocytes. Each exon and the exon-intron boundaries of CASR were amplified by PCR using the primers previously described (6, 22). After amplification, the PCR products were purified and sequenced directly with an ABI PRISM Dye Terminator Cycle Kit and an ABI 373A automated fluorescent sequencer (PE Applied Biosystems).

Plasmid construction and site-directed mutagenesis

Human CASR cDNA was obtained from NPS Pharmaceuticals and inserted into a human expression vector (pCDNA3.1; Invitrogen). A modified CASR cDNA was constructed, containing a FLAG epitope tag, YKDDDDK, and inserted between amino acids 22 and 23 just after the signal peptide in the CASR NH2-terminal region. Site-directed mutagenesis to generate mutant CASR cDNAs containing point mutations was performed by Prime STAR Mutagenesis (Takara) using pCDNA-CASR expression vector. All constructs were confirmed by DNA sequencing before transfection. We also constructed R648X, a loss-of-function mutant (23, 24), and A843E, a constitutively active mutant (25).

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were cultured in 5% CO_2 at 37°C in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. HEK293 cells were transiently transfected using TransIT-LT1 Transfection Reagent (Mirus) according to the manufacturer's protocol and cultured at 37°C in 5% CO_2 for 48 hours before experiments.

Assay of CASR signal transduction of Gi-MAPK pathway

Activation of G protein-coupled signal transduction by wild-type or mutant CASRs was investigated with the use of Western blot analysis and luciferase assays. MAPK activity induced by

activation of the CASR by extracellular Ca^{2+} was assessed by examining the phosphorylation of ERK1/2 with Western blot analysis as previously reported (26, 27). In brief, HEK293 cells were transiently transfected with wild-type or mutant CASR cDNAs in 100-mm dishes. Twenty-four hours later, the cells were serum-starved in DMEM containing 0.5 mM $CaCl_2$ for 18–24 hours. At the end of incubation, cells were treated with DMEM with 2.0 mM $CaCl_2$ for 10 minutes. Cell lysates were obtained using lysis buffer supplemented with protease inhibitor cocktail (Thermo Scientific). Whole protein extracts were separated by SDS-PAGE (4–12% gel) and analyzed by Western blotting with an anti-phospho-p44/42 ERK polyclonal antibody (Cell Signaling Technology) or p44/42 ERK antibody (Cell Signaling Technology). Quantitative comparison of ERK1/2 phosphorylation was performed using Multi Gauge version 2.0 (Fujifilm). The ratios of the phosphorylated to nonphosphorylated EKR1/2 signals were calculated. To evaluate the activity of CASR with luciferase assays, we used pGL4.33, which is a reporter vector containing a serum response element (Promega) (28, 29). HEK293 cells were plated in 12-well plates to 70% confluency and transiently transfected using TransIT-LT1 Transfection Reagent with 0.5 μg wild-type or mutant or empty vector, 0.5 μg pGL4.33, and β-galactosidase vector. The following day, cells were serum-starved in DMEM containing 0.5 mM $CaCl_2$ for 18–22 hours and cultured in various concentrations of $CaCl_2$ ranging from 0.5–30 mM for 30 minutes. Cell extracts were prepared, and luciferase assays were performed. Luciferase measurements were divided by the respective β-galactosidase activity to control for transfection efficiency. All transfections were performed in triplicate, and the experiment was repeated 3 times.

Fluorescence analysis and confocal microscopy

HEK293 cells cultured on glass coverslips were transfected with either 1 μg wild-type or mutant FLAG-tagged CASR cDNA. Forty-eight hours after transfection, transfected HEK293 cells were fixed for 20 minutes in 4% (vol/vol) paraformaldehyde/PBS. Cell surface CASR was stained with anti-FLAG mouse M2 monoclonal antibody (Sigma-Aldrich) at 4°C overnight. Cell surface CASR was labeled with fluorescein isothiocyanate goat antimouse IgG (Invitrogen). Cells were then permeabilized with 1% Triton X-100 at room temperature for 20 minutes. Intracellular CASR was stained with Alexa Fluor 555 goat antimouse IgG (Invitrogen) at 4°C overnight. Cells were examined on a FV10i confocal microscope (Olympus).

Biotinylation of cell-surface forms of the CASR and Western blot analysis

To extract proteins on the cell surface, we used a cell surface protein isolation kit (Thermo Scientific). HEK293 cells were plated in 75-cm^2 flasks to 70% confluency and transiently transfected using a TransIT-LT1 Transfection Reagent with 9 μg wild-type or mutant FLAG-tagged CASR cDNA. Forty-eight hours after transfection, cells were harvested for plasma membrane preparation according to the manufacturer's protocol. The biotinylated proteins were subsequently subjected to electrophoresis in 4–12% SDS-PAGE and then electrotransferred to nitrocellulose membranes. The presence of CASR was detected with the anti-FLAG antibody and visualized by ECL Plus Western Blotting Detection Reagents (GE Healthcare).

Fluorescence-activated cell sorting (FACS) analysis

To quantify the extent of cell surface expression of CASR, fluorescence intensity was measured using flow cytometry. HEK293 cells grown on six-well plates were transiently transfected with 2 μg wild-type or mutant FLAG-tagged CASR cDNA and 0.5 μg pAcGFP1-Mem (Clontech-Takara), which results in labeling of the plasma membrane. Forty-eight hours after transfection, cells were gently scraped with Accutase-Enzyme Cell Detachment Medium (eBioscience). Detached cells were washed with ice-cold PBS, and 10^6 cells were incubated for 20 minutes at 4°C with 1:500 diluted anti-FLAG antibody. Cells were washed with PBS and incubated for another 20 minutes with 1:250 diluted PE Goat antimouse IgG (BioLegend) at 4°C. Cells were washed with PBS and immediately analyzed on a BD FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed with Cellquest (Becton Dickinson). All transfections were performed in triplicate, and the experiment was repeated 3 times. Cell surface expression levels of CASR were divided by the expression levels of green fluorescent protein-positive to control for transfection efficiency.

Effect of allosteric modulators (NPS R-568 and NPS 2143)

NPS R-568 was a kind gift from Kirin Pharmaceutical, and NPS 2143 was purchased from Hangzhou Hetd Industry. For cell treatments, stock solutions of NSP R-568 or NPS 2143 in 1% ethanol were added to DMEM. In these experiments, we used 2.0 mM extracellular calcium because this concentration is considered to be within the physiological range as reported previously (20). Cells were incubated at 100 nM, 1 μM, and 10 μM for 6 hours before harvest. Control solutions contained equivalent volumes of ethanol, which did not influence the results of functional assay and flow cytometry (data not shown). To reflect the situation in vivo, the effect of allosteric modulators was also studied with cotransfection of each mutant and wild-type. Furthermore, to evaluate the time-resolved effect of NPS R-568 and NPS 2143, HEK293 cells transfected with wild-type or mutant CASR were incubated with 1 μM allosteric modulators for 0, 1, 2, 4, 6, 9, or 12 hours and treated with 2.0 mM Ca^{2+} for 30 minutes before luciferase assay.

Statistics

The mean EC_{50} (the effective concentration of agonist giving one-half of the maximal response) for the wild-type or mutant receptor in response to increasing concentrations of Ca^{2+} was calculated using GraphPad Prism (GraphPad Software, Inc).

Values are represented as the mean ± SEM. Statistical analysis was carried out using Mann-Whitney *U* test.

Results

Patients

The clinical details of the patients are summarized in Table 1. As shown in Table 1, high serum calcium levels, absolute or relative low urinary calcium excretion, and family history of hypercalcemia led to the diagnosis of FHH. Patients with ADH had low serum calcium levels despite inappropriately low intact PTH levels and high

Table 1. Biochemical Characteristics and CASR Mutations of FHH and ADH Patients

	FHH		ADH		
Patient no.	**1**	**2**	**3**	**4**	**5**
Age at manifestation	10 y	1 y 5 mo	Neonatal	Neonatal	Neonatal
Symptoms	(—)	(—)	(—)	(—)	Seizure
Serum total calcium, mg/dL (mmol/L)[a]	11.3 (2.82)	12.8 (3.19)	7.1 (1.78)	7.5 (1.87)	7.5 (1.87)
Serum phosphate, mg/dL (mmol/L)[b]	2.9 (0.94)	4.1 (1.39)	ND	6.0	ND
Intact PTH, pg/mL[c]	87.4	20	39	5	4–12
Urinary Ca/Cre, mg/mg (mmol/mmol)[d]	0.10	0.05–0.09 (0.16–0.29)	0.2–0.4 (0.66–1.31)	0.3–0.5 (0.98–1.64)	0.22–0.47 (0.71–1.55)
Family history	Mother (hypercalcemia)	Mother, grandmother (hypercalcemia)	Mother (hypocalcemia)	Father (hypocalcemia)	(—)
Mutations of *CASR*	Exon 3	Exon 4	Exon 3	Exon 6	Exon 7
	ECD	ECD	ECD	ECD	TMD
	c.328G>A	c.514A>G	c.365C>G	c.1706C>A	c.2516T>C
	p.A110T	p.R172G	p.S122C	p.P569H	p. I839T

Abbreviations: ND, not determined; (—), no symptoms. Normal ranges are in metric units with System International units in parentheses.

[a] Normal reference ranges: neonate, 8.20–9.76 (2.05–2.44); infant, 8.56–9.96 (2.13–2.49); ages 9–11 years, 8.90–9.83 (2.22–2.45).

[b] Normal reference ranges: neonate, 4.25–6.45 (1.37–2.08); infant, 3.29–5.17 (1.06–1.67); ages 9–11 years, 3.22–4.42 (1.04–1.43).

[c] Normal reference range is 10–60.

[d] Normal ranges of urinary Ca/Cre: 0.05–0.25 (0.16–0.82).

urinary calcium excretion. Two patients with ADH had a family history of hypocalcemia. Clinical course and family history were briefly mentioned.

Patients of FHH

Patient 1

At age 10 years, patient 1 was referred to the local hospital for abdominal pain. Laboratory examination accidentally revealed hypercalcemia (12.1 mg/dL; normal reference range, 8.90–9.83 mg/dL for the age), elevated intact PTH levels (87.4 pg/mL; normal reference range, 10–60 pg/mL), and relative hypocalciuria (urinary calcium/creatinine [Ca/Cre], 0.10 mg/mg; normal reference range, 0.05–0.25 mg/mg). Family analysis showed that his mother also had hypercalcemia (10.5 mg/dL) and relative hypocalciuria (urinary Ca/Cre, 0.05 mg/mg). His grandmother was also found to have hypercalcemia.

Patient 2

At age 1 year, patient 2 was hospitalized with pneumonia. At this time, he was found to have hypercalcemia (12.8 mg/dL; normal reference range, 8.56–9.96 mg/dL for the age), a normal PTH level (20 pg/mL), and relative hypocalciuria (urinary Ca/Cre, 0.05–0.09 mg/mg). His mother also showed hypercalcemia (12–13 mg/dL) and relative hypocalciuria (urinary Ca/Cre, 0.05–0.1 mg/mg).

These findings and family histories indicated that the diagnosis of FHH was suspected for patients 1 and 2.

Patients of ADH

Patient 3

Patient 3 was born via normal vaginal delivery without asphyxia. During the neonatal period, he was diagnosed as having transient tachypnea of the newborn. At this time, laboratory examination showed that his serum calcium level was low (7.1 mg/dL; normal reference range, 8.20–9.76 mg/dL for the age) with a normal intact PTH level (39 pg/mL). Treatment of 1α-hydroxyvitamin D3 was begun. Thereafter, relative hypercalciuria (urinary Ca/Cre, 0.2–0.4 mg/mg) was continued during the administration of 1α-hydroxyvitamin D3 despite hypocalcemia. Hydrochlorothiazide was added to maintain serum calcium level and reduce urinary calcium excretion. Family analysis showed that his mother also had hypocalcemia (7.8 mg/dL) with relatively high urinary calcium excretion (urinary Ca/Cre, 0.4 mg/mg). She has been also treated with 1α-hydroxyvitamin D3 and hydrochlorothiazide.

Patient 4

Patient 4 was diagnosed as having hyperbilirubinemia and was treated with phototherapy in the neonatal period. At this time, routine laboratory examination demonstrated hypocalcemia (7.5 mg/dL). Further examination indicated that his serum intact PTH was low (5 pg/mL) and his urinary calcium excretion was increased (urinary Ca/Cre, 0.3–0.5 mg/mg) despite hypocalcemia. His father had several tetanic episodes since childhood, and laboratory data indicated hypocalcemia and relatively high urinary calcium excretion (data not available).

Patient 5

Patient 5 was evaluated because of afebrile seizure in the neonatal period. Laboratory examination showed hypocalcemia (7.5 mg/dL), low intact PTH level (4–12 pg/mL), and elevated urinary calcium excretion (urinary Ca/

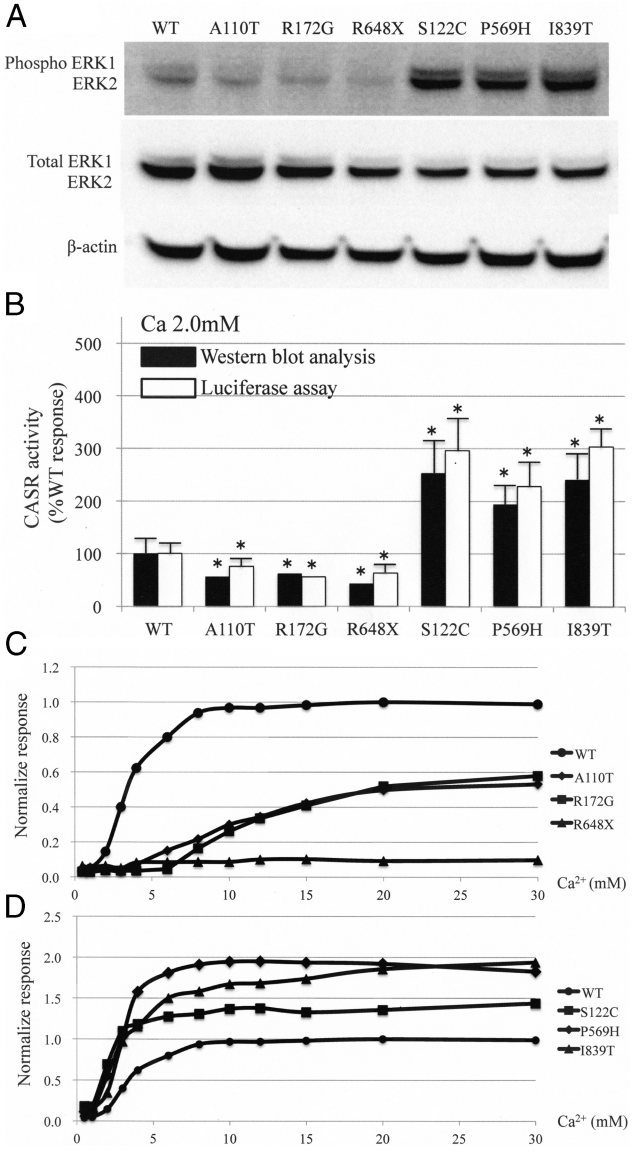

Figure 1. Activation of the Gi-MAPK pathway by the wild-type and mutant CASRs. A, Phosphorylation of ERK1/2 activated extracellular 2.0 mM Ca^{2+} in HEK293 cells transiently transfected with either the wild-type or mutant CASR cDNA. Western blots were stained with antibodies against either phospho- or total ERK1/2 and β-actin as a loading control. B, Black bars indicate the quantification of ERK1/2 phosphorylation of the wild-type or mutant CASRs in response to 2.0 mM Ca^{2+}. White bars indicate the Gi-MAPK pathway activity of wild-type or mutant CASRs using luciferase assay (pGL4.33 vector) in response to 2.0 mM Ca^{2+}. The ERK1/2 phosphorylation activities examined by Western blot analysis and Gi-MAPK activations examined by a luciferase assay system of each mutant were compared with that of wild-type CASR using the Mann-Whitney *U* test. *, $P < .05$. The activities of A110T and R172G were significantly decreased compared with that of the wild-type, and the activities of S122C, P569H, and I839T were significantly increased compared with that of the wild-type in both examinations. C and D, Extracellular calcium-evoked increase in Gi-MAPK pathway activity in HEK293 cells transiently transfected with either the wild-type or mutant CASR cDNA and pGL4.33 vector. C, Dose-response curves of Gi-MAPK pathway activity in response to a stepwise increase of extracellular calcium of the wild-type and inactivating mutants (A110T, R172G, and R648X). D, Dose-response curves of Gi-MAPK pathway activity of the wild-type and activating mutants (S122C, P569H, I839T).

Cre, 0.22–0.47). His parents and siblings had normal serum calcium levels.

Based on these findings, the diagnosis of ADH was suspected in patients 3, 4, and 5.

Mutations of CASR

Direct sequence analysis of *CASR* in FHH patients identified 1 novel heterozygous mutation (c.328G>A, p.A110T) and another heterozygous mutation (c.514A>G, p.R172G) which had been reported previously (www.casrdb.mcgill.ca). In ADH patients, 3 novel heterozygous mutations (c.365C>G, p.S122C; c.1706C>A, p.P569H; and c.2516T>C, p.I839T) were found.

Signal transduction of mutant CASRs

We investigated the activation of the Gi-MAPK pathway of the mutant CASRs in response to extracellular Ca^{2+} (2.0 mM) by Western blot analysis (Figure 1, A and B). The ERK1/2 phosphorylation activities of 2 mutants (A110T and R172G) were significantly decreased, and those of 3 mutants (S122C, P569H, and I83T) were significantly increased compared with that of wild-type in response to 2.0 mM extracellular Ca^{2+} (Figure 1, A and B). We also analyzed Gi-MAPK activation using a luciferase assay system. The results of the luciferase assay system were in agreement with the results of the Western blot analysis (Figure 1B). Therefore, we further investigated the activation of the Gi-MAPK pathway in response to various concentrations of extracellular Ca^{2+} using the luciferase assay system. The Gi-MAPK pathway dose-response curves of 2 mutants (A110T and R172G) identified in FHH patients were significantly right-shifted (Figure 1C), with increases in EC_{50} ranging from 7.75 to 7.87 mM, compared with 2.95 mM for the wild-type (Table 2).

In contrast, all 3 mutants (S122C, P569H, and I839T) in the ADH patients showed significantly left-shifted Gi-MAPK pathway dose-response curves (Figure 1D), with decreased EC_{50} ranging from 1.62 to 2.79 mM, compared with 2.95 mM for the wild-type (Table 2). These findings confirmed that A110T and R172G were inactivating mutants and that S122C, P569H, and I839T were activating mutants.

Table 2. Summary of in Vitro Data

	EC_{50}, mM	Cell Surface Expression, %
Wild-type	2.95 ± 0.29	100.0 ± 19.1
A110T	7.75 ± 2.27	105.3 ± 13.8
R172G	7.87 ± 2.43	166.7 ± 10.9
S122C	1.62 ± 0.39	138.5 ± 31.4
P569H	2.02 ± 0.59	74.6 ± 6.3
I839T	2.79 ± 0.55	88.2 ± 5.7

Cell surface expression of mutant CASR

To analyze the cell surface expression of CASR mutants, immunofluorescence analysis was performed on HEK293 cells. All activating and inactivating mutants were expressed at the cell surface, although R648X was poorly expressed in the plasma membrane (Figure 2A), in agreement with a previous study (24). In addition, intracellular staining of S122C and R172G was less than that of wild-type (Figure 2A).

Western blot of plasma membrane proteins isolated from transiently transfected wild-type or mutant FLAG-tagged CASR cDNAs except R648X demonstrated both

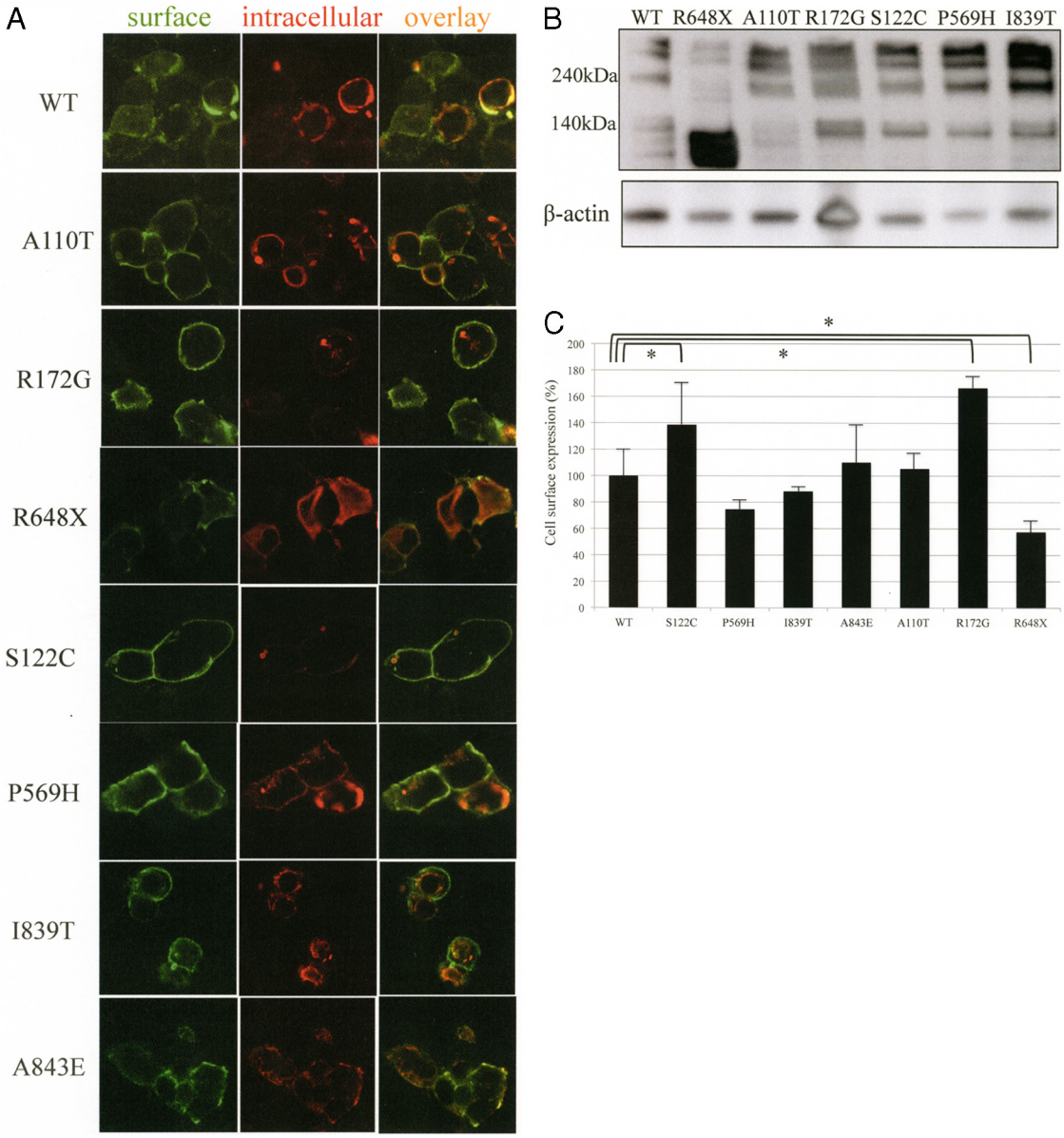

Figure 2. Cell surface localization of the wild-type and mutant CASRs. A, Fluorescence immunocytochemistry and confocal microscopy were performed on HEK293 cells transiently transfected with wild-type or mutant CASRs. Left panels, The cell surface expression of each CASR was detected (green staining). Middle panels, The intracellular CASR was visualized after being permeabilized (red staining). Merging of the left and middle panels is shown in the right panels. Activating and inactivating mutants except R648X were expressed at the cell surface, whereas membrane expression of R648X was attenuated and intracellular expression was increased relative to the wild-type. B, Western blot analysis of biotinylated cell surface protein from HEK293 cells transiently transfected with either the wild-type or mutant CASR cDNA. The wild-type and mutant CASRs exist in both monomeric and dimeric forms. The 140-kDa band is the monomeric form of CASR, and the high-molecular-mass bands (>240 kDa) are the dimeric or oligomeric forms. The nonsense mutation, R648X, which is predicted to encode a truncated protein lacking the TMD and intracellular tail shows a smaller band of about 100 kDa. C, Quantification of the wild-type and mutant CASR cell surface expression by flow cytometry. Graph represents averaged results of 9 independent experiments, with all data normalized to the cell surface expression of wild-type CASR. The cell surface expression level of each mutant was compared with wild-type CASR using the Mann-Whitney *U* test. *, $P < .05$. The amount of cell surface expression of S122C and R172G was significantly increased compared with that of the wild-type; however, the plasma membrane expression levels of P569H, I839T, A843E, and A110T were almost identical to that of the wild-type. WT, wild-type.

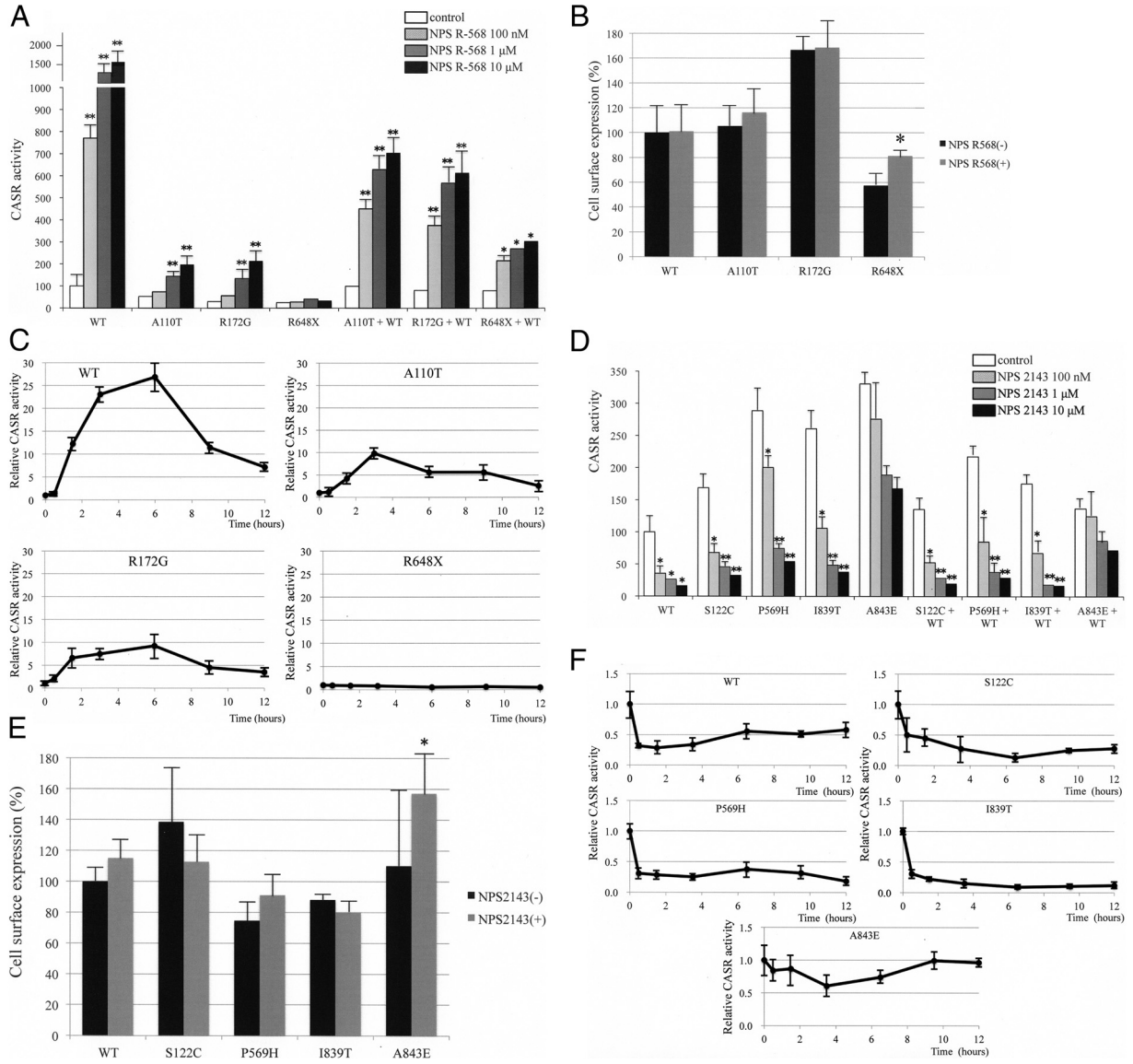

Figure 3. The effect of NPS R-568 (A–C) and NPS 2143 (D–F) on mutant CASRs. A, Functional rescue of NPS R-568 (100 nM, 1 μM, and 10 μM) in the presence of 2.0 mM extracellular Ca^{2+} in the Gi-MAPK pathway in HEK293 cells transiently transfected with wild-type or inactivating mutant CASRs. All data were normalized to the activity of wild-type CASR without NPS R-568 in 2.0 mM extracellular Ca^{2+}. *, $P < .05$; **, $P < .01$ indicates a significant difference in Gi-MAPK pathway activity between control and NPS R-568 treatment. White bars indicate activity in the absence of NPS R-568, and dotted, gray, and black bars indicate activity in the presence of NPS R-568. B, Alternation of cell surface expression by NPS R-568 treatment in the presence of 2.0 mM extracellular calcium. All data are normalized to cell surface expression of wild-type CASR in ethanol. The levels of cell surface expression of wild-type and each mutant except R648X did not increase after treatment with NPS R-568. The cell surface expression of R648X was significantly increased by NPS R-568. *, $P < .05$. C, Time-resolved rescue of wild-type and inactivating mutant (A110T, R172G, and R648X) CASR by NPS R-568. HEK293 cells were transiently transfected with wild-type or inactivating mutant CASRs and incubated with 1 μM NPS R-568 for 1, 2, 4, 6, 9, and 12 hours in response to 2.0 mM extracellular calcium. Maximal functional rescue was observed after 4–6 hours of treatment in wild-type and each inactivating mutant except R648X. NPS R-568 did not rescue the activity of R648X. D, Functional suppression of NPS 2143 (100 nM, 1 μM, and 10 μM) in the presence of 2.0 mM extracellular Ca^{2+} in the Gi-MAPK pathway. *, $P < .05$; **, $P < .01$ indicates a significant difference in the Gi-MAPK pathway activity between control and NPS 2143 treatment. White bars indicate activity in the absence of NPS 2143, and dotted, gray, and black bars indicate activity in the presence of NPS 2143. All data are normalized to the activity of wild-type CASR without NPS 2143. For wild-type, S122C, P569H, and I839T, significant differences were observed between control and NPS 2143 treatment in Gi-MAPK pathways in a concentration-dependent manner; however, the degree of suppression by NPS 2143 in A843E was milder than other mutants. In cotransfection experiments, NPS 2143 also suppressed signal transduction by each mutant. E, Changes in cell surface expression by NPS 2143 treatment in the presence of 2.0 mM extracellular calcium. All data were normalized to the cell surface expression of wild-type CASR in ethanol. Although cell surface expression of the wild-type and each mutant was unaffected by treatment with NPS 2143, cell surface expression levels of A843E were significantly increased by NPS 2143. *, $P < .05$. F, Time-resolved suppression of wild-type and activating mutant (S122C, P569H, I839T, and A843E) CASRs by NPS 2143. HEK293 cells were transiently transfected with wild-type or mutant CASR cDNA and incubated for 1, 2, 4, 6, 9, and 12 hours with 1 μM NPS 2143 in response to 2.0 mM extracellular calcium. Maximal suppression occurred 1 hour after treatment and lasted for at least 12 hours in wild-type and each activating mutant except A843E. The degree of suppression in A843E was less than other mutants and recovered to the same extent as the activity in the absence of NPS 2143 after 10 hours.

140-kDa monomeric bands and high-molecular-mass bands (>240 kDa), corresponding to the dimeric or oligomeric forms, respectively, in the wild-type and other mutant CASRs, as reported previously (27, 29) (Figure 2B).

To further quantify CASR cell surface expression, FACS analysis was performed. Although cell surface expression levels of the activating S122C mutant (138.5 ± 31.4%) and the inactivating R172G mutant (166.7 ± 10.9%) was significantly increased compared with that of the wild-type (100.0 ± 19.1%), the expression levels of P569H (74.6 ± 6.3%), I839T (88.2 ± 5.7%), A843E (110.1 ± 29.2%), and A110T (105.3 ± 13.8%) did not differ from that of the wild-type (Figure 2C). The cell surface expression level of R648X (57.5 ± 9.3%) was significantly decreased. The results were in agreement with that of the immunofluorescent staining.

The effect of NPS R-568 on activating mutant CASR

To study whether or not NPS R-568 could rescue the activity of the inactivating CASR mutants, HEK293 cells expressing wild-type CASR or the A110T, R172G, or R648X mutants were treated with NPS R-568. As shown in Figure 3A, in response to 2.0 mM calcium, NPS R-568 significantly increased Gi-MAPK signaling activity mediated by wild-type, A110T, and R172G CASRs in a dose-dependent manner.

As expected, the activity of R648X could not be rescued. When wild-type CASR and the A110T or R172G mutants were cotransfected, NPS R-568 rescued the impaired activity of each mutant. We next analyzed cell surface expression levels after treatment with NPS R-568. As shown in Figure 3B, although expression of the wild-type, A110T, and R172G was not affected by NPS R-568, cell surface expression of R648X was significantly increased. In a time course experiment, maximal functional rescue of NPS R-568 is observed after 4- 6-hour treatment, and the degree of rescue was gradually reduced with time (Figure 3C). The signal transduction capacity of the wild-type was greater than that of the inactivating mutants at each time point.

The effect NPS 2143 on inactivating mutant CASR

We next analyzed the effect of NPS 2143. NPS 2143 significantly suppressed the activity of wild-type CASR as well as the enhanced Gi-MAPK pathway activity of S122C, P569H, I839T, and A834E in a dose-dependent manner in response to 2.0 mM extracellular calcium (Figure 3D). In the presence of NPS 2143, the activities of S122C, P569H, and I839T were lower than the wild-type in the absence of NPS 2143. In experiments in which wild-type and each mutant were cotransfected, NPS 2143 also suppressed the enhanced activation of the mutant CASRs. In the case of A843E, NPS 2143 suppressed the enhanced activity; however, the degree of the suppression was less pronounced than that of other activating mutations (Figure 3D). We next analyzed CASR cell surface expression levels in the presence of NPS 2143. Although cell surface expression of the wild-type, S122C, P569H, and I839T CASRs was not affected by treatment with NPS 2143, cell surface expression of A843E increased after treatment with NPS 2143 (Figure 3E). The suppression of NPS 2143 was observed after a 1-hour treatment and lasted at least 12 hours (Figure 3F). By contrast, in the case of A843E, the degree of suppression by NPS 2143 was mild compared with other mutants, and the effect of NPS 2143 disappeared after 12 hours of treatment.

Discussion

Both hypoparathyroidism and ADH are characterized by hypocalcemia with inappropriate low PTH levels, although relative or absolute hypocalciuria is observed only in patients with ADH, the treatment of which with vitamin D leads to nephrocalcinosis and renal impairment. Therefore, the identification of a CASR-activating mutation clearly distinguishes ADH patients from hypoparathyroidism. Similarly, genetic analysis of the *CASR* gene is useful for distinguishing FHH patients from hyperparathyroid patients. Genetic analysis of the *CASR* gene is therefore useful for the diagnosis of ADH and FHH. Not all changes in *CASR* gene sequence alter the function of the receptor, however, and novel mutations in *CASR* require functional analysis to determine their consequence for CASR function. In our study, A110T and R172G were shown to be inactivating, and S122C, P569H, and I839T were shown to be activating.

It has been reported that the cell surface expression levels of loss- and gain-of-function mutants are variable (13, 14, 20, 30). In our study, whereas the expression level of A110T was similar to that of wild-type, the expression levels of R172G were significantly increased over wild-type. In gain-of-function mutants, although the plasma membrane expression levels of P569H, I839T, and A843E did not differ from wild-type, the cell surface expression levels of S122C were significantly greater than that of wild-type.

We tested whether the calcimimetic NPS R-568 and the calcilytic NPS 2143 function as positive and negative allosteric modulators of the mutant CASRs, respectively. Our results show that NPS R-568 can rescue the activity of A110T and R172G in a dose-dependent manner. Fur-

thermore, the cell surface expression levels of the 2 mutants were unaltered after treatment with NPS R-568. Rus et al (31) reported that NPS R-568 can rescue the activity of inactivating mutants expressed on the cell surface and that the rescue is due to increased efficacy of Ca^{2+} receptor activation. It has also been reported that increased expression levels of mutant receptors at the plasma membrane after treatment with NPS R568 is one mechanism by which mutant receptor function is rescued (13, 14). In our experiments, functional rescue is likely to be attributable to the former mechanism. Furthermore, our results showed that NPS 2143 robustly suppressed the activity of the gain-of-function mutants S122C, P569H, and I839T. After treatment with NPS 2143, cell surface expression levels of wild-type CASR and the S122C, P569H, and I839T mutants were unchanged. Therefore, we have demonstrated that functional suppression is caused by decreasing sensitivity to Ca^{2+} rather than by decreased amounts of mutant receptor at the plasma membrane. In contrast to our results, Huang and Breitwieser (13) have reported that NPS 2143 decreased cell surface expression of the gain-of-function mutants F128L, E191K, Q681H, and F788C. Although the exact reason for this difference is not known, it may be attributable to specific effects of NPS 2143 on different gain-of-function mutants. We have shown that A843E was less sensitive to NPS 2143 than S122C, P569H, and I839T with regard to signal suppression. However, because the effect of NPS 2143 on the plasma membrane expression levels of A843E differed from its effect on other activating mutants, the increased cell surface expression levels of A843E may offset the reduction of signal transduction activity by NPS 2143. Because the exact mechanism involved in the increased cell surface expression levels of A843E by NPS 2143 is not yet known, this must be studied further.

Letz et al (20) have reported the effects of NPS 2143 on several activating mutants. According to their study, 1 μM NPS 2143 was fully effective for G830S, partially effective for T151R, E767Q, and A844T, and ineffective for P221L. Taken together, it is plausible that the effectiveness of NPS 2143 varies among different mutants. We also investigated the dose and time-course effect of NPS R-568 and NPS 2143. The effect of NPS R-568 and NPS 2143 was dose-dependent, in agreement with previous reports (12, 20, 32). With regard to the time-course effect, the effect of NPS 2143 was observed longer than that of NPS R-568. The suppressive effect of NPS 2143 to wild-type and inactivating mutant CASRs, with the exception of A843E, continued at least 12 hours after treatment of NPS 2143. The study of the time-resolved effects of allosteric modulators is important for evaluation of their pharmacological and clinical potential. Allosteric modulators potentiate or antagonize CASR activity by binding receptors that were located on the plasma membrane. Additionally, it has been reported that these compounds can bind to immature receptors and affect their stability and degradation (13, 14). This may explain the time-course effect of NSP R-568 and NPS 2143. Furthermore, the different time courses may also reflect the stability of the drugs themselves.

In conclusion, we have identified 3 gain-of-function and 2 loss-of-function mutations in CASR. We have also demonstrated that NPS R-568 is able to rescue the activity of the loss-of-function mutations and that NPS 2143 can suppress the enhanced activity of the gain-of-function mutations.

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