CASE REPORT

Two novel mutations of the calcium-sensing receptor gene affecting the same amino acid position lead to opposite phenotypes and reveal the importance of p.N802 on receptor activity

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Abstract

Objective: Gain-of-function mutations of the calcium-sensing receptor (CASR) gene have been identified in patients with sporadic or familial autosomal dominant hypocalcemia (ADH). Inactivating mutations of the CASR gene cause familial hypocalciuric hypercalcemia (FHH). Here, we report two novel CASR mutations affecting the same amino acid (p.N802); one causes ADH and the other atypical FHH.

Patients and methods: The first patient, an 11-year-old girl suffering from hypocalcemia, developed nephrocalcinosis when she was only 5 years old. The second patient is a 30-year-old woman who presented with mild hypercalcemia. PCR amplification of CASR coding exons and direct sequencing of PCR products were used to identify mutations. Site-directed mutagenesis was used to generate mutated CASR cDNAs in an expression plasmid. Using the MAPK assay system and transient transfection of Cos-7 cells with wild-type (WT) and mutated CASR, we studied the responses of these mutated receptors to extracellular Ca^{2+} and to the negative allosteric CASR modulator, NPS2143.

Results: Two heterozygous missense mutations (p.N802I and p.N802S) affecting a residue in the sixth transmembrane domain of CASR were identified. In functional tests, the response of the p.N802S mutant to calcium was typical of an inactivating mutation. However, the p.N802I mutant had 70% of the maximally stimulated WT receptor activity even in the absence of extracellular calcium. This constitutive activity was only partially inhibited by the inhibitor, NPS2143.

Conclusions: The asparagine at amino acid position 802 appears to be essential for the activity of the CASR protein and is implicated in the mechanism of CASR signaling.

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Introduction

The calcium-sensing receptor (CASR) is a G protein-coupled receptor (GPCR) central to regulating extracellular calcium ion concentrations. CASR is abundant in tissues and cells involved in calcium homeostasis, such as the parathyroid glands and cells lining the kidney tubule (1). It is responsive to very small changes in the circulating calcium concentration and, once activated, it inhibits parathyroid hormone (PTH) secretion and renal tubule calcium reabsorption. The importance of this receptor as a 'calciostat' has been emphasized by the identification of naturally occurring mutations in the CASR

gene: inactivating and activating mutations cause hypercalcemic or hypocalcemic disorders respectively (2, 3, 4, 5). Individuals heterozygous for loss-of-function mutations of CASR develop familial hypocalciuric hypercalcemia (FHH) and homozygotes present with neonatal severe hyperparathyroidism (NSHPT). By contrast, familial/sporadic autosomal dominant hypocalcemia (ADH) is caused by heterozygous gain-of-function CASR mutations (6). The clinical phenotype of these patients is usually mild or in some cases asymptomatic and the biological phenotype associates hypocalcemia with normal but unadapted persistent calciuria and low PTH levels (6, 7).

More than 131 naturally occurring loss-of-function mutations and 71 gain-of-function mutations have been reported (Fig. 1 and the online database at (<http://www.casrdb.mcgill.ca>)) (8). CASR has a large extracellular amino terminal domain (ECD) (606 amino acids corresponding to around 56% of total amino acids), seven transmembrane domains (TMD) (23% of protein), and an intracellular carboxy-terminal domain (ICD) (21% of protein). Known loss-of-function mutations are scattered throughout the protein, but gain-of-function mutations are nonrandomly distributed (9): 52.1% map in the ECD (10) and many clusters between residues 116 and 131; 42.3% map in the TMDs with clusters in TMD6 (23.8% of TMD mutants) and in TMD7 (30%); and only 5.6% are in the ICD (11). The identification and analysis of these naturally occurring mutations have helped elucidate the structure and function of this receptor.

We report a study of two novel mutations, p.N802I and p.N802S, of the CASR gene, affecting the amino acid position 802. This amino acid position is in TMD6 in which seven other mutants have already been described. Intriguingly, the p.N802I and p.N802S mutants behaved differently in functional tests.

Materials and methods

Patients

The patients were recruited through the National French Calcium-Phosphorus Network. Informed consent was obtained from the patients and their families, according to the guidelines of the French Bioethics Authority for the protection of human studies.

DNA amplification and sequence analysis

Genomic DNA was prepared from patient peripheral blood mononuclear cells with an Easy-DNA extraction kit (Invitrogen). Exons 2–7 of the CASR gene, comprising the entire coding sequence, were amplified by PCR. Both strands of the PCR products were sequenced using an Applied Biosystems mode ABI 3130xl automated sequencer with the Big Dye Terminator Cycle Sequencing kit v.1.1 (Applied Biosystems, Foster City, CA, USA).

Site-directed mutagenesis

The cDNA for human CASR between the HindIII and XhoI restriction sites of the expression vector pCR3.1 (pCR3.1/hCASR) was provided by Prof. Anne Lienhardt-Roussie (CHU, Limoges, France). The Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) was used according to the manufacturer's directions. The sequence of all constructs was verified by sequencing.

Transient transfection of Cos-7 cells with human CASR cDNA

Cos-7 cells were propagated in DMEM containing 10% heat-inactivated FCS (Eurobio, Courtaboeuf, France) in 75 cm² flasks under 5% CO₂ at 37 °C. For transfection, Cos-7 cells were cultured in six-well plates and transfected with wild-type (WT) or mutant pCR3.1/hCASR DNA and the Elk-1 *trans*-reporting system (Stratagene) using PolyFect transfection reagents (Qiagen). Twenty-four hours after transfection, cells were starved of serum in DMEM containing no CaCl₂ for 8 h and then cultured for 16 h in the presence of various concentrations of CaCl₂ from 0 to 4 mM, with and without 300 nM NPS-2143. Cells were washed in PBS, lysed on ice, and MAPK activity was assayed.

MAPK activity

Exogenous MAPK activity induced by CASR activation was assessed as described previously (12). In brief, a *trans*-reporting system (Stratagene) was used to measure the activity of Elk-1, an ETS domain transcription factor targeted by MAPK pathways. Luciferase activity in 25 µl aliquots of cell lysate was measured after addition of 50 µl D-luciferin using a Turner Biosystems 20/20⁺ luminometer (Promega). Luciferase activity was normalized to β-galactosidase activity.

Results

Case 1

Proband 1 (Table 1) is now an 11-year-old girl. Her medical history started at 8 months of age, when she experienced severe seizures associated with marked hypocalcemia (1.24 mmol/l) due to primitive hypoparathyroidism (0.66 pmol/l). Treatment with 1-α hydroxylated vitamin D was initiated and helped normalize the serum calcium level (2.17 mmol/l). However, when she was 5 years old, she developed nephrocalcinosis (calcium/creatinine 1.35 mmol/mmol) and mild renal failure; the dose of 1-α hydroxylated vitamin D was decreased and calcium supplementation was added. At age 6 years, dental enamel abnormalities were noticed and a severe episode of fatigue was observed. At age 7 years, plasma magnesium deficiency was detected. These various findings strongly suggested the involvement of a CASR gain-of-function mutation. The family history was negative for any similar clinical profiles (Fig. 2a).

Direct sequence analysis of PCR-amplified CASR exons identified a heterozygous mutation (c.2405A → T) in exon 7 leading to an amino acid substitution (p.N802I) in the protein. This change was not found in any of the 100 CASR gene alleles from 50 unrelated normal individuals. No other mutations were identified.

Table 1 Biochemical analyses at various ages in Proband 1 carrying the p.N802I mutation.

Age	Serum calcium (mmol/l) ref (2.10–2.55)	PTH (pmol/l) ref (1.1–5.8)	Calcium/creatinine (mmol/mmol) ref (<1)	Natremia (mmol/l) ref (136–145)	Kaliemia (mmol/l) ref (3.2–5)
8 months	1.24	0.66	0.005	—	—
5 years 2 months	2.17	0.43	—	140	3.7
6 years	2.05	0.32	1.35	—	—
6 years 6 months	1.87	—	—	—	—
7 years 2 months	2.02	0.43	0.71	—	—
7 years 9 months	2.18	0.85	1.5	138	4

DNA samples from the other family members were not available, but the absence of a similar clinical picture in the family suggested that this *CASR* mutation occurred *de novo* in Proband 1.

Case 2

Proband 2 (Table 2), a 30-year-old woman, had been suffering from tiredness for a long time. A biochemical check-up showed a mild elevated serum calcium level (2.69 mmol/l) but an inappropriate high level of PTH (7.98 pmol/l). Her mother and sister were also tested: results for the mother were normal, whereas the sister showed a normal high serum calcium level (2.48 mmol/l), a high calciuria value (7.75 mmol/day), and a very high level of PTH (13.1 pmol/l) (Table 2 and Fig. 2b).

Nucleotide sequencing identified a novel heterozygous mutation (c.2405A→G) in exon 7 leading to a p.N802S amino acid substitution in the protein. This change was not found in 100 *CASR* gene alleles from 50 unrelated normal individuals. No other mutations were identified. The mutation was also present in the affected sister but not in the asymptomatic mother, who had normal *CASR* sequences. DNA samples from the father were not available but the presence of the mutation in two daughters strongly suggests that the father was a carrier of the mutation.

Functional analysis of WT and mutant receptors

The responses of the mutant and WT receptors to extracellular calcium were assessed using a MAPK pathway *trans*-reporting system (Fig. 3). WT *CASR* cDNA was transiently expressed in Cos-7 cells: for the WT-*CASR*, the EC_{50} of Ca^{2+} was 2.22 ± 0.16 mM (mean $\pm$ S.E.M.; $n=4$; Fig. 3a), and, as expected, there was no cellular response when WT-*CASR* was not stimulated (extracellular calcium at 0 mM); the activity progressively increased with increasing Ca^{2+} concentration to a plateau (100% activity) at 4 mM Ca^{2+} (Fig. 3a, bold line).

Most *CASR* with activating mutations show a leftward shift in their dose-response curve relative to

that of the WT. The mutation p.E241K, already described by Christie *et al.* (13) and also detected in one of our patients suffering from hypocalcemia, was used as a reference (Fig. 3a, dotted line). Mutant p.N802I (Proband 1) showed a different profile: at 0 mM Ca^{2+} , it displayed 70% of the maximal WT activity, suggesting that the mutation had a constitutive activating effect even in the absence of extracellular calcium stimulation.

Furthermore, the activity of mutant p.N802I also increased up to 114% of the maximum WT activity at 4 mM Ca^{2+} concentration. Therefore, it was sensitive to Ca^{2+} concentration in addition to its constitutive activation.

To our knowledge, only one other *CASR* mutation has been described that causes constitutive activation: the p.A843E mutation in TMD7 (14). In order to compare p.N802I with p.A843E properly, we tested them side by side in our system. The p.A843E mutation shows a strong constitutive activation, but its response to extracellular calcium differs from that of the p.N802I mutation (Fig. 4). In the absence of Ca^{2+} , p.A843E displays 150% of the maximal WT activity and this value is stable (around 155%) at all Ca^{2+} concentrations. Thus, it is possible to say that, in addition to its constitutive activation, p.N802I seems to remain sensitive to Ca^{2+} concentration, which is not the case for p.A843E.

Mutant p.N802S (Proband 2) showed impaired *CASR* function, confirming the clinical diagnosis of FHH. The dose-response curve was right-shifted relative to the WT receptor, consistent with an inactivating mutation (Fig. 3a).

Figure 2 Pedigrees of the two families studied, (a) Case 1, (b) Case 2. The probands are indicated by an arrow. The genotypes were determined by mutation analysis. N corresponds to the normal allele. Clinical data are given in Materials and methods section.

Table 2 Biochemical analyses of family members and of Proband 2 carrying the p.N802S mutation.

Patient	Serum calcium (mmol/l) ref (2.10–2.55)	PTH (pmol/l) ref (1.1–5.8)	Urinary calcium (mmol/day) ref (1.25–6.25)	25-OH Vit D (nmol/l) ref (>75)
Proband (II-1)	2.69	7.98	4.05	36.5
Mother (I-1)	2.38	5	1.12	–
Sister (II-2)	2.48	13.09	7.75	34

Treatment of CASR mutants with the calcilytic agent NPS-2143

To test whether the calcilytic agent NPS-2143 could reduce CASR mutant p.N802I activity at calcium concentrations within the physiological range, we stimulated cells with various extracellular calcium concentration in the presence or absence of 300 nM NPS-2143 (Fig. 3b). In Cos-7 cells expressing WT CASR, NPS-2143 abolished the tested CASR activity, demonstrating that it is active in our system (dashed line). The same phenomenon was observed for p.N802S CASR, which already presented a weak level of activity (not shown). The CASR activity of the constitutively active mutant, p.N802I, was lower in the presence than in the absence of NPS-2143; however, NPS-2143 did not completely inhibit its activity, such that at all extracellular calcium concentrations the CASR mutant p.N802I activity was about half that of the maximum observed for the WT enzyme in the absence of NPS-2143.

Discussion

Two novel CASR mutations of the same amino acid (p.N802) lead to opposite symptoms. The heterozygous missense mutation p.N802I, a novel gain-of-function CASR mutation, causes hypercalciuric hypocalcemia whereas the heterozygous missense mutation p.N802S, an inactivating CASR mutation, results in mild hypercalcemia. This type of phenomenon has rarely been described for CASR and these observations suggest that this amino acid is central to ligand binding and/or receptor activation.

Other amino acid mutations of CASR have been reported to induce opposite effects: p. P221L is an activating mutation whereas p.P221Q and p.P221S inactivate CASR (15, 16, 17, 18); similarly, p.E297D is activating and p.E297K is inactivating (4, 19, 20), and p.L173F is activating and p.L173P is inactivating (21, 22); all these mutations are located in the ECD. These amino acid positions are presumably located in regions involved in ligand binding (Ca²⁺), as suggested by Silve *et al.* (20). In the TMD, the p.A843V mutation leads to CASR inhibition, whereas p.A843E leads to constitutive activation of the CASR, independent of calcium concentration and ECD structure. This mutation probably locks the TMD in an active conformation (14). The p.N802 mutations we describe

here are localized at the beginning of the sixth TMD of CASR (Fig. 1) (23). This region appears to be a hot spot for gain-of-function mutations. Indeed, eight of the 71 known gain-of-function mutations (11.3%) of CASR map within this region (which is only around 2.4% of the entire CASR protein), suggesting that this region has an important role in CASR activity.

A model of GPCR activation is that there is a dynamic equilibrium between two states of the GPCR: a conformation favoring G protein coupling (R*) and one that does not favor G protein coupling (24). Many naturally occurring activating missense mutations have

Figure 3 Extracellular calcium-evoked increases in MAPK activity in Cos cells transiently transfected with a MAPK *trans*-reporting system and wild-type (WT) or mutant p.N802I or p.N802S or p.E241K CASR cDNAs without (a) or with (b) 300 nM NPS-2143 treatment. Values reported are mean $\pm$ S.E.M. of four experiments. All responses are normalized to the maximum response of WT human CASR.

Figure 4 Extracellular calcium-evoked increases in MAPK activity in Cos cells transiently transfected with a MAPK *trans*-reporting system and wild-type (WT) or mutant p.N802I or p.A843E CASR cDNAs. Values reported are mean $\pm$ S.E.M. of four experiments. All responses are normalized to the maximum response of WT human CASR.

been identified in various GPCRs in numerous diseases. Analysis of agonist-independent constitutive activating mutations led to the idea that mutation of these residues disrupts the normal inhibitory constraints and favors the formation of R*. Most such mutations map to TMD6, implicating this region in GPCR activation (see 25) (for review). In contrast, most activating CASR mutations result in increased sensitivity to Ca²⁺ rather than true constitutive activation. Of the 71 activating CASR mutations identified to date, 37 map in the ECD, a region known to be important for Ca²⁺ binding. Missense mutations of the ECD may therefore lead to inappropriate CASR activation by increasing the affinity for Ca²⁺. Similarly, the 30 mutations localized within the CASR TMD or extra/intracellular loops also exhibit enhanced sensitivity to Ca²⁺ rather than constitutive activation. To our knowledge, only two CASR mutations have been described that cause constitutive activation rather than enhanced Ca²⁺ sensitivity: the p.A843E mutation in TMD7 (14) and the p.N802I mutation we report here in TMD6. Our head-to-head comparison between these two constitutive mutations shows that while both of them present a very strong constitutive activation in the absence of Ca²⁺ (67 and 150% for p.N802I and p.A843E respectively), their responses to extracellular calcium differ (Fig. 4). Only p.N802I seems to remain sensitive to Ca²⁺ concentration which is not the case for p.A843E.

The p.N802 residue is highly conserved in mammals (*Pan troglodytes*, *Mus musculus*, *Rattus norvegicus*, *Canis lupus familiaris*, and *Bos taurus*) and also in other animal species (*Gallus gallus* and *Danio rerio*). Interestingly, this amino acid is also conserved in other non-CASR GPCRs, including the human metabotropic glutamate receptors (mGluR), consistent with it being functionally important. We therefore suggest that p.N802I, without Ca²⁺

binding, disrupts the inhibitory constraints that prevent G protein coupling. It is possible that the mutation affects side-chain packing in TMD6 or contacts with residues in other TM helices, for example p.A843. Such changes could alter the configuration of one or more intracellular loops in such a way that it favors G protein coupling. However, the structure of CASR has not been accurately determined, so these suggestions remain speculative.

We show that the p.N802I mutant is only partially sensitive to NPS-2143, an allosteric modulator of the CASR: in the presence of NPS-2143, the response of p.N802I CASR is lowered (to 40% from 67% of the WT value, in the absence of calcium) and it becomes insensitive to the calcium concentration but retains constitutive activity (around 40%). This is unlike all other tested activating mutations: NPS-2143 decreases but does not abolish sensitivity to calcium (9). This may indirectly indicate that p.N802 is an important amino acid involved in the G protein signaling.

The phenotype of Proband 1 (p.N802I) was severe. She presented with symptoms from age 8 months and required treatment with 1- α hydroxylated vitamin D for marked hypocalcemia and had iatrogenic nephrocalcinosis by age 5 years. It proved difficult to normalize serum calcium level (Table 1). This clinical presentation is consistent with the corresponding mutant's response curve to calcium and the strength of its constitutive activation. While Bartter syndrome has been observed in ADH patients presenting strong activation mutations, such as p.A843E or p.L125P (14, 26), this syndrome has not been observed in our patient (normal natremia and kaliemia, Table 1). By contrast, the phenotype of Proband 2 (p.N802S) was very mild and she was diagnosed only when she was 30 years old. One should note the atypical presentation of FHH in this family, which could have been misdiagnosed as primary hyperparathyroidism if DNA testing and functional studies had not secured the diagnosis. Indeed, inappropriate high PTH level and normal calciuria to hypercalciuria are observed in the proband and in her sister. Similar observations have already been made in FHH families (3, 27, 28). High PTH level could be explained by vitamin D deficiency, as vitamin D supplementation has been shown to reduce serum PTH levels to the normal range in FHH patients (29). Hypercalcemia with hypercalciuria has already been observed in a Swedish family presenting p.F881L mutation in CASR (28). Thereafter, several other mutations (p.T100I, p.L650P, p.V689M (27), p.Q926R, and p.D1005N (30)) have been associated with atypical 'FHH' presentations including hypercalciuria. One hypothesis could be that various biochemical phenotypes in FHH patients could be correlated with specific genotypes (17) and that mutations may not have the same impact on the distinct signaling pathways regulated by CASR (MAPK pathways activation vs PI-PLC for instance) and in different tissues

(parathyroid glands vs cortical thick ascending limb). It could be interesting to test this hypothesis on a large panel of classical and atypical FHH patients.

These findings suggest that the sixth TMD, and specifically p.N802, have a major role in CASR activation. Our study illustrates the value of precise characterization of the activation profile of CASR mutants. This allows phenotype–genotype correlations, which may help select adequate treatment for the patient.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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