CASE REPORT

A novel mutation of the primary protein kinase C phosphorylation site in the calcium-sensing receptor causes autosomal dominant hypocalcemia

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Abstract

Objective: The calcium-sensing receptor (CASR) is a key controller of calcium homeostasis by regulating parathyroid hormone (PTH) secretion and renal calcium reabsorption. CASR T888 is a protein kinase C (PKC) phosphorylation site in the receptor’s intracellular domain that has previously been identified as a critical negative regulator of CASR downstream signaling in vitro, but whose importance in vivo is unknown.

Case report: The proband presented with mild symptomatic hypocalcemia following treatment for nephrotic syndrome due to minimal change glomerulonephropathy. Laboratory tests revealed inappropriately normal PTH concentrations and relative hypercalciuria typical of autosomal dominant hypocalcemia. His asymptomatic father had similar laboratory test results.

Design and methods: The CASR gene was sequenced. To investigate the molecular consequences of CASR T888M mutation, site-directed mutagenesis was used to modify the wild-type (wt)-CASR gene, with the resulting mutant being transfected transiently into HEK-293 cells.

Results: A novel CASR missense mutation, T888M, was identified in both cases. The CASR T888M mutant exhibited enhanced sensitivity to extracellular calcium concentration, both for intracellular calcium (Ca2+) mobilization and for ERK phosphorylation, despite having unaltered levels of cell surface expression. Furthermore, CASR T888M elicited sustained Ca2+ mobilization rather than high frequency Ca2+ oscillations, and, unlike the wt-CASR, the response was resistant to acute inhibition by the PKC activator, phorbol 12-myristate 13-acetate.

Conclusions: The clinical and functional data provide the first genotype–phenotype correlation for a mutation at T888, indicating its critical physiological importance in CASR signaling. Thus, CASR T888 represents a functionally important, inhibitory phosphorylation site that contributes to the control of PTH secretion.

Introduction

The calcium-sensing receptor (CASR; MIM 601199) is a G protein-coupled receptor that is a key controller of extracellular calcium (Ca2+) homeostasis. CASR is highly expressed in the chief cells of the parathyroid gland, but is also expressed in other tissues including the thyroid, kidney, gastrointestinal tract, and bone. Both loss-of-function and gain-of-function CASR mutations have been described (1–3).

Heterozygous loss-of-function mutations generally result in familial hypocalciuric hypercalcemia, and homozygous mutations result in neonatal severe hyperparathyroidism. The former is characterized by mild and the latter by severe hypercalcemia, together with inappropriately normal or frankly elevated parathyroid hormone (PTH) concentrations (1–3). Conversely, gain-of-function mutations resulting in impairment of PTH secretion and renal calcium and magnesium reabsorption manifest as autosomal dominant hypocalcemia (ADH) (1–3). More than fifty activating mutations have been described to date. These are collated at www.casrdb.mcgill.ca and have been the subject of recent reviews (4, 5). Although often familial, de novo mutations are not uncommon, with one case study of 19 unrelated cases of isolated hypoparathyroidism identifying eight individuals with distinct CASR mutations (6).
The phenotype of ADH is characterized by persistent hypocalcemia with clinical features that vary from asymptomatic through to asthenia, muscle cramps, paresthesia, carpopedal spasm, and even seizures, in combination with inappropriately low concentrations of PTH and relative hypercalcuria (2, 6). While in the hypocalcemic range, the serum total corrected calcium is almost never <1.20 mmol/l, with other suggestive features being a PTH within the normal range (i.e. around 1.0–6.0 pmol/l), serum magnesium ≤0.70 mmol/l and a urine calcium:creatinine ratio >0.30 mmol/mmol (2). Owing to the relative hypercalcuria, ADH is associated with an increased risk of nephrocalcinosis and renal impairment, particularly with the use of active vitamin D analogs (2, 6, 7). 1,25-dihydroxyvitamin D (1.25(OH)2D) has been shown to up-regulate renal CASR expression, and increased expression of activated CASR may result in worsening hypercalcuria (8, 9).

While most mutations responsible for ADH are located in the extracellular regions of the CASR, several are located in the intracellular domain where they may influence trafficking, surface expression, and receptor signaling rather than agonist binding. An intracellular CASR residue known to be critical for the control of CASR activity in vitro is the protein kinase C (PKC) phosphorylation site threonine-888 (10–15); however, the in vivo effects of mutations at this site have not been described previously. In this study, we describe a family with ADH due to a novel T888M missense mutation and thus demonstrate the importance of this residue to CASR signaling in vivo.

Case report

The proband was a 24-year-old Caucasian male who was referred for evaluation of hypocalcemia. Eight months previously, he had developed nephrotic syndrome due to biopsy-confirmed minimal change glomerulonephropathy that readily responded to prednisolone therapy. He had also been treated with calcium carbonate 1500 mg and cholecalciferol 1000 IU/day for osteoporosis prophylaxis. The treating nephrologist noted hypocalcemia after the proteinuria and hypoalbuminemia had resolved.

There was a history of intermittent muscle cramps and carpal spasms since childhood that had improved on one occasion following a short course of calcium and magnesium supplements. These symptoms recurred during his illness, but settled after calcium and cholecalciferol were started.

There was no history of neck trauma or surgery, hearing impairment, seizures, or febrile convulsions. There was no known family history of hypocalcemia. Examination was unremarkable apart from the presence of several psoriatic plaques consistent with a history of psoriasis from childhood.

Blood tests taken 2 years prior to his presentation with nephrotic syndrome showed that the total serum calcium corrected for albumin was low at 1.96 mmol/l (reference range 2.15–2.55), albumin was normal at 46 g/l (reference range 35–50), and phosphate was high at 1.80 mmol/l (reference range 0.81–1.45). No action had been taken at that time. Blood tests performed during the hypoalbuminemic period associated with the nephrotic syndrome (serum albumin <20 g/l) demonstrated apparently normal corrected calcium concentrations. However, the total corrected calcium concentration was clearly below the lower limit of normal (values between 1.97 and 2.18 mmol/l) once the serum albumin normalized.

Investigations performed following referral showed that serum-ionized calcium was low at 1.05 mmol/l (reference range 1.15–1.35), serum magnesium was normal at 0.9 mmol/l (reference range 0.7–1.1), intact PTH was inappropriately normal (4.7 pmol/l; reference range 1.0–7.0), 25-hydroxyvitamin D (25OHD) was normal (132 nmol/l; reference range 50–150), but 1.25(OH)2D was elevated (213 pmol/l; reference range 40–153). Similar results were obtained on a repeat blood test 6 days later. The elevated 1.25(OH)2D concentration was further investigated with chest X-ray and serum angiotensin-converting enzyme concentration, which were normal, and Quantiferon-TB Gold Assay for Mycobacterium tuberculosis, which was elevated. Subsequent serial morning sputum samples were negative for M. tuberculosis, and a tuberculin skin test was negative. Infectious disease review concluded that the patient had a minimal likelihood of M. tuberculosis infection, and a cause for the high 1.25(OH)2D level was not identified.

A random urinary calcium:creatinine ratio was 0.48 mmol/mmol (reference range <0.4 in normocalcemic individuals) with a paired corrected serum calcium of 2.18 mmol/l. On a subsequent 24-h urinary specimen, the calcium:creatinine ratio was 0.15 mmol/mmol with a paired serum calcium of 2.00 mmol/l. Both samples were taken while the patient was taking calcium supplements.

Screening of relatives (father, mother, brother, and father's siblings), all of whom were asymptomatic, showed that the patient’s father was also hypocalcemic, but not other family members. The father’s serum total corrected calcium was 2.01 mmol/l and serum-ionized calcium was 1.06 mmol/l, with an inappropriately normal serum PTH of 1.5 pmol/l and high urinary calcium:creatinine ratio of 0.43 mmol/mmol. Serum phosphate (1.3 mmol/l), magnesium (0.9 mmol/l), and 25OHD (103 nmol/l) were normal. Unlike the index case, 1.25(OH)2D was normal (111 pmol/l). These findings led us to suspect the presence of a heterozygous gain-of-function mutation of the CASR in the proband and his father consistent with a diagnosis of ADH. Informed consent for further testing and publication of the findings was obtained.
Methods

Biochemical tests

Routine laboratory analytes were assayed on a Beckman Coulter UniCel DxI 800 analyzer (Beckman Coulter Diagnostics, Brea, CA, USA). Calcitonin and PTH were measured on a Siemens Immulite 2000 analyzer (Siemens Medical Solutions Diagnostics Ltd, Malvern, PA, USA). Serum 25OHDL was measured with ultra-performance liquid chromatography followed by tandem mass spectrometry (16), and serum 1,25(OH)2D3 was measured with an immunoassay (Diasorin, North Ryde, NSW, Australia).

PCR product amplification and sequencing

DNA sequencing of the CASR gene was performed on extracted genomic DNA with an automated sequencer (ABI Prism; Perkin-Elmer Corp., Wellesley, MA, USA) as previously described (17).

Site-directed mutagenesis and cell culture

The T888M mutation was introduced by site-directed mutagenesis using the QuikChange (Stratagene, Agilent Technologies, Stockport, Cheshire, UK) method with the wild-type (wt) human parathyroid CASR in pcDNA3.1 as the template. HEK-293 cells were transiently transfected (FuGENE-6, Roche Diagnostics) with pcDNA3.1 as the template. HEK-293 cells were transiently transfected with FLAG-CASR-induced changes in intracellular calcium (Ca2+) sensitivity of the receptors.

Cell surface expression

HEK-293 cells were transfected transiently with FLAG-tagged (extracellular) CASR constructs. After 48 h, the cells were incubated with mouse monoclonal DDDDK tag antibody (G10; 1 μg/ml; Abcam, Sapphire Bioscience, Waterloo, Australia) in DMEM (10% fetal bovine serum (FBS)) for 2 h (4 °C with gentle rocking), and then washed and collected with ice-cold PBS and pelleted by centrifugation. Cells were then incubated with HRP-conjugated goat anti-mouse IgG antibody (1:5000; Sigma–Aldrich) in DMEM (10% FBS) for 1 h (4 °C with vigorous shaking). Cells were pelleted and washed as before and then incubated with 3,3′,5′,5′-tetramethylbenzidine (Sigma–Aldrich) for 20 min (in the dark with rocking), and the reaction was then stopped with an equal volume of 1.0 M HCl. Cells were pelleted, and then the absorbances of the supernatants were read in triplicate at 450 nm. Absorbances were corrected for background using an untransfected control and expressed as percentages of the cell surface expression of a wt control.

ERK phosphorylation

HEK-293 cells transfected transiently with CASR were assayed for ERK phosphorylation as previously described (19). Briefly, cells were exposed to buffer containing various Ca2+ concentrations at 37 °C for 10 min (or to 4 mM Ca2+ for various time points up to 20 min) prior to lysis on ice in RIPA buffer supplemented with protease and phosphatase inhibitors. ERK phosphorylation was then determined by semi-quantitative immunoblotting using a phosho-specific antibody (Pierce, Fisher Scientific UK Ltd, Loughborough, UK).

Presentation of data and statistical analysis

The data are presented as means ± S.E.M., and statistical significance between datasets was determined by Student’s unpaired or paired t-test (GraphPad Prism, La Jolla, CA, USA).

Results

Sequence analysis of exon 7 of the CASR genomic DNA in both the proband and his father revealed a heterozygous substitution of cytosine with thymidine at position 2663 (CASR:c.[2663C>T]) (Fig. 1). This mutation was predicted by PolyPhen (prediction of functional effect of human nsSNPs; http://genetics.bwh.harvard.edu/PPH/) to have a position-specific independent counts (PSIC) score of 1.711, and
To investigate the functional consequence of the CASR<sup>T888M</sup> mutation in vitro, the mutation was recapitulated by mutating the wt-CASR cDNA. CASR<sup>T888M</sup> exhibited an apparent increase in Ca<sup>2+</sup> sensitivity relative to wt-CASR, and the EC<sub>50</sub> for Ca<sup>2+</sup> decreased significantly (Fig. 2; wt, 3.0 ± 0.1 mM; T888M, 2.5 ± 0.2 mM; *P* < 0.05 by unpaired *t*-test of log EC<sub>50</sub> values). Western blotting revealed similar expression of protein bands consistent with the well-recognized 140 kDa core-glycosylated and 160 kDa mature receptors (7, 12–15, 20) in both sets of transfected cells (Fig. 2C). In addition, there was no difference in cell surface expression between wt-CASR and CASR<sup>T888M</sup>-tagged constructs in their respective extracellular domains.

Next, to investigate the consequence of the T888M mutation on the recognized inhibitory effect of phorbol ester treatment, wt-CASR- and CASR<sup>T888M</sup>-transfected cells were exposed to raised Ca<sup>2+</sup> concentration (4 mM) in the absence and then in the presence of 1 μM phorbol 12-myristate 13-acetate (PMA), a PKC stimulator. For wt-CASR, high Ca<sup>2+</sup> concentration induced rapid Ca<sup>2+</sup> oscillations that were abolished within 2 min of PMA cotreatment (Fig. 3A; in the absence of PMA, Ca<sup>2+</sup> oscillations continued for at least 10 min (12)). In contrast, CASR<sup>T888M</sup> elicited mostly sustained Ca<sup>2+</sup> mobilization that exhibited a time-dependent decay and resistance to PMA-induced inhibition (Fig. 3B).

Finally, the apparent increase in Ca<sup>2+</sup> sensitivity seen with CASR<sup>T888M</sup> with respect to Ca<sup>2+</sup> mobilization (Fig. 2) was also observed in experiments examining the effect of CASR activation on ERK phosphorylation (Fig. 4; WT, 3.4 ± 0.2 mM; T888M, 2.6 ± 0.2 mM; *P* < 0.01 by paired *t*-test of log EC<sub>50</sub> values). The effects of 4 mM Ca<sup>2+</sup> on each receptor as a function of time were broadly similar (up to 20 min) though with a small but significant increase in ERK phosphorylation observed after 1 min for CASR<sup>T888M</sup> receptor (Fig. 4C). No further differences were observed in the response time courses.
regained or retained the responsiveness to high Ca\(^{2+}\). Following removal of PMA and a brief recovery phase, cells (not shown). Median responses are shown and are representative signal inhibition. (A) High Ca\(^{2+}\) elicit CASR-mediated Ca\(^{2+}\) responses of wt-CASR but had an impaired effect on CASR\(^{T888M}\). Following removal of PMA and a brief recovery phase, cells regained or retained the responsiveness to high Ca\(^{2+}\) concentration (4 mM) was used to elicit CASR-mediated CASR\(^{T888M}\) mobilization resulting in oscillatory signaling for the wt-CASR and sustained Ca\(^{2+}\) signaling for CASR\(^{T888M}\). Cotreatment with PMA (1 µM) abolished the responses of wt-CASR but had an impaired effect on CASR\(^{T888M}\). Following removal of PMA and a brief recovery phase, cells regained or retained the responsiveness to high Ca\(^{2+}\) concentration (not shown). Median responses are shown and are representative of three separate coverslips. (B) The Ca\(^{2+}\) \(^{2+}\) response to 4 mM Ca\(^{2+}\) (average area under the 350/380 ‘global’ cluster trace per minute) during exposure to PMA expressed as a percentage of the average response per minute over the 3 min prior to exposure. CASR\(^{T888M}\) was largely unresponsive to PMA, whereas for wt-CASR, PMA virtually abolished the response (P<0.05 by unpaired t-test).

**Discussion**

ADH in humans is a well-recognized consequence of activating mutations of the CASR, and a similar phenotype has been reported in the Nuf mouse, which is heterozygous for the CASR activating mutation L723Q (21). ADH is typically associated with increased Ca\(^{2+}\) sensitivity either from enhanced coupling between the receptor’s extracellular Ca\(^{2+}\)-binding domains and intracellular signaling domains, or from enhanced trafficking and surface expression (1). The findings reported here demonstrate that ADH can also arise when an inbuilt mechanism that uncouples the receptor from its intracellular signaling pathways is irreversibly disabled.

It has been shown previously that PTH secretion is increased by PKC activation in parathyroid cells exposed to moderate or high Ca\(^{2+}\) concentrations that would otherwise suppress PTH secretion (22–27). Cloning of the CASR revealed PKC consensus sequences in its intracellular domain, and it was proposed that phosphorylation of one or more of these sites might uncouple the CASR from its downstream signaling pathway, thereby disinhibiting PTH secretion (28).

Of the five predicted PKC consensus sites in the human CASR C-terminus, T888 is functionally the most important in terms of phosphatidylinositol phospholipase C-dependent signaling in HEK-293 cells (13). Bai et al. (13) demonstrated this using site-directed mutagenesis of the five sites, finding that mutation of T888 substantially altered receptor sensitivity and responsiveness to PMA. In contrast, mutation of PKC sites T646 and S794 in the intracellular loops was without effect, and mutation of the intracellular domain residues S895 or S915 had only a small effect on receptor sensitivity and responsiveness to PMA (9). Specifically, mutation of CASR\(^{T888}\) to certain non-phosphorylatable residues (e.g. alanine and valine) increased apparent Ca\(^{2+}\) sensitivity of the CASR, whereas phosphomimetic mutations (e.g. aspartate and glutamate) attenuated Ca\(^{2+}\) sensitivity (13, 14). Rather than altering Ca\(^{2+}\) sensitivity per se as an extracellular mutation might, mutation of CASR\(^{T888}\) modulates receptor-induced signaling, preventing CASR-mediated Ca\(^{2+}\) mobilization when phosphorylated (as mimicked by CASR\(^{T888D}\)), but permitting sustained CASR-mediated Ca\(^{2+}\) mobilization when phosphorylation is prevented as in the case of CASR\(^{T888D}\) in vitro (12–15) or CASR\(^{T888M}\) in vivo as reported in this study.

As previous studies on the significance of T888 for the control of CASR signaling were conducted using heterologous CASR expression in HEK-293 cells, it has been uncertain whether the CASR\(^{T888}\) residue exerts a critical function in vivo. Herein, we describe the clinical consequence of CASR\(^{T888}\) mutation in humans. As predicted by the in vitro studies, we found that CASR\(^{T888M}\)
is a gain-of-function mutation in vivo, resulting in ADH featuring inappropriately suppressed serum PTH levels, hypercalciuria, and hypocalcemia. To our knowledge, this is the first clinical case report of a mutation disrupting T888 phosphorylation. It confirms the importance of T888 in CASR function and also supports the current model for its role in CASR signaling.

Live-cell imaging reveals that stimulation of the wt-CASR elicits Ca\textsuperscript{2+} oscillations (29–32) in a PKC-sensitive manner, with PKC activation reducing the frequency of Ca\textsuperscript{2+} oscillations (30, 31). This suggests that CASR-mediated Ca\textsuperscript{2+} oscillations result from negative feedback provided by PKC-mediated phosphorylation at T888, producing dynamic phosphorylation and dephosphorylation of CASR\textsuperscript{T888} (31). In addition, we show here for the first time that downstream ERK phosphorylation is affected similar to Ca\textsuperscript{2+} mobilization by CASR\textsuperscript{T888} mutation, with CASR\textsuperscript{T888M} exhibiting greater Ca\textsuperscript{2+} sensitivity (Fig. 4) but without increasing cell surface expression of a FLAG-tagged receptor.

Recently, the use of a phospho-specific antibody against an epitope that included the phospho form of CASR\textsuperscript{T888} demonstrated PKC-mediated phosphorylation of the mature CASR in response to receptor activation, leading to feedback phosphorylation of CASR\textsuperscript{T888} (12). At lower levels of stimulation (i.e. 2 mM Ca\textsuperscript{2+}), CASR\textsuperscript{M} produced low frequency Ca\textsuperscript{2+} oscillations, whereas the relatively high concentration of 3 mM Ca\textsuperscript{2+} elicited mostly sustained responses (Figs 1 and 2) similar to that reported previously for CASR\textsuperscript{T888} (8). We have also recently shown that the pattern of CASR-elicited Ca\textsuperscript{2+} mobilization is dependent on receptor-stimulated dephosphorylation of CASR\textsuperscript{T888}, as it permits either the next Ca\textsuperscript{2+} oscillation or even a sustained response (15). In the light of these previous in vitro data, the current clinical study indicates that loss of the CASR\textsuperscript{T888} phosphorylation site via a T to M mutation results in gain-of-function at the level of the parathyroid gland and in turn, a significant disturbance of whole body calcium homeostasis. Furthermore, it is consistent with the presence of some degree of basal phosphorylation of the wt-CASR by PKC in vivo, which tonically suppresses the activity of the receptor under normocalcemic conditions. Loss of the capacity of PKC to phosphorylate CASR\textsuperscript{T888M} should remove this inhibitory action, thereby increasing the activity of the mutant CASR and producing the biochemical phenotype of ADH.

Interestingly, 1.25(OH)\textsubscript{2}D\textsubscript{3} was initially elevated in the index case, but was normal in his father. The conversion of 25OHD to its active metabolite 1.25(OH)\textsubscript{2}D\textsubscript{3} is mediated by 1\alpha-hydroxylase, a crucial regulatory enzyme of predominantly renal origin. Hypocalcemia stimulates renal 1\alpha -hydroxylase activity independently of PTH and hypophosphatemia (33), and although the CASR is expressed in renal proximal tubule cells (34), it is not yet clear whether it mediates the effect of low serum Ca\textsuperscript{2+} concentration on 1\alpha-hydroxylase.

The current findings point either to the possibility of a CASR-independent mechanism or to a CASR signaling pathway whose sensitivity to serum Ca\textsuperscript{2+} concentration is not enhanced by the T888M mutation. In conclusion, we have described a family with ADH due to a novel CASR mutation, T888M. This case provides the first reported genotype–phenotype correlation of a mutation at this site, confirms previous in vitro findings that identified T888 as an important phosphorylation site in the CASR, and supports the current model for its role in CASR-mediated intracellular signaling.

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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