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.

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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 hypercalciuria (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 $1.0\text{--}6.0$ pmol/l), serum magnesium ≤ 0.70 mmol/l and a urine calcium:creatinine ratio >0.30 mmol/mmol (2). Owing to the relative hypercalciuria, 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 ($1,25(\text{OH})_2\text{D}$) has been shown to up-regulate renal CASR expression, and increased expression of activated CASR may result in worsening hypercalciuria (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 $2.15\text{--}2.55$), albumin was normal at 46 g/l (reference range $35\text{--}50$), and phosphate was high at 1.80 mmol/l (reference range $0.81\text{--}1.45$). 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 $1.15\text{--}1.35$), serum magnesium was normal at 0.9 mmol/l (reference range $0.7\text{--}1.1$), intact PTH was inappropriately normal (4.7 pmol/l; reference range $1.0\text{--}7.0$), 25-hydroxyvitamin D (25OHD) was normal (132 nmol/l; reference range $50\text{--}150$), but $1,25(\text{OH})_2\text{D}$ was elevated (213 pmol/l; reference range $40\text{--}153$). Similar results were obtained on a repeat blood test 6 days later. The elevated $1,25(\text{OH})_2\text{D}$ 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 $1,25(\text{OH})_2\text{D}$ 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, $1,25(\text{OH})_2\text{D}$ 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 25OHD was measured with ultra-performance liquid chromatography followed by tandem mass spectrometry (16), and serum 1,25(OH)₂D 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 wt-CASR or CASR^{T888M} and grown on glass coverslips in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen Ltd).

Total cellular CASR protein expression

Protein expression was demonstrated by western blotting as described previously, using an anti-CASR mouse MAB (amino acids 214–235 of human CASR; Affinity Bioreagents, Golden, CO, USA) (18, 19). Briefly, cells were lysed in detergent-containing buffer supplemented with protease inhibitors and *N*-ethylmaleimide (1 mM), the lysate was centrifuged at 10 000 *g* (10 min, 4 °C), and the supernatant was solubilized in Laemmli buffer at 65 °C (3 min).

Intracellular calcium assay

CASR-induced changes in intracellular calcium (Ca_i²⁺) concentration were measured by epifluorescence microscopy as described previously (11, 12) using cells loaded with Fura-2 (Invitrogen). CASR-transfected cells were then incubated at room temperature in Experimental Buffer (20 mM HEPES (pH 7.4), 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, and 5.5 mM glucose) and exposed to increasing

concentrations of Ca_o²⁺ to establish the relative Ca_o²⁺ sensitivities 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 Ca_o²⁺ concentrations at 37 °C for 10 min (or to 4 mM Ca_o²⁺ 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 phospho-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

Figure 1 Representative sequence analysis of exon 7 of the *CASR* in the proband and his father (B). The wild-type sequence (NM_000388.2) is included for comparative purposes (A). The heterozygous substitution of cytosine with thymidine at position 2663 (*CASR*:c.[2663C>T]) is predicted *in silico* to result in the substitution of a methionine for threonine at position 888 (p.Thr888Met) and thus abolish the phosphorylation site. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-10-0907>.

modeling of the effect on the amino acid sequence of the *CASR* protein predicted a substitution of methionine for threonine at position 888 (p.T888M) that would abolish a major intracellular PKC phosphorylation site (12).

Functional characterization of the *CASR*^{T888M} mutation in HEK-293 cells

To investigate the functional consequence of the *CASR*^{T888M} mutation *in vitro*, the mutation was recapitulated by mutating the *wt-CASR* cDNA. *CASR*^{T888M} exhibited an apparent increase in Ca_o^{2+} sensitivity relative to *wt-CASR*, and the EC_{50} for Ca_o^{2+} 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_{50} 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* ($100 \pm 6.4\%$) and *CASR*^{T888M} ($93.9 \pm 4.9\%$) FLAG-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*^{T888M}-transfected

cells were exposed to raised Ca_o^{2+} 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_o^{2+} concentration induced rapid Ca_i^{2+} oscillations that were abolished within 2 min of PMA cotreatment (Fig. 3A; in the absence of PMA, Ca_i^{2+} oscillations continued for at least 10 min (12)). In contrast, *CASR*^{T888M} elicited mostly sustained Ca_i^{2+} mobilization that exhibited a time-dependent decay and resistance to PMA-induced inhibition (Fig. 3B).

Finally, the apparent increase in Ca_o^{2+} sensitivity seen with *CASR*^{T888M} with respect to Ca_i^{2+} 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_{50} values). The effects of 4 mM Ca_o^{2+} 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*^{T888M} receptor (Fig. 4C). No further differences were observed in the response time courses.

Figure 2 Extracellular Ca_o^{2+} -induced Ca_i^{2+} mobilization in HEK-293 cells transfected with either wild-type *CASR* or *CASR*^{T888M}. (A) Fura2-loaded HEK-293 cells (i) wild-type *CASR*; (ii) *CASR*^{T888M} were treated with increasing Ca_o^{2+} concentrations (0.5–10 mM), and the resulting changes in Ca_i^{2+} concentration (350/380 ratio) were measured by single cell microfluorimetry. Representative traces show Ca_i^{2+} changes in two single cells and a 'global' cluster of all of the responsive cells in the field of view. Results are representative of a minimum of nine coverslips from two independent transfections. (B) Concentration-effect curve for Ca_o^{2+} -induced Ca_i^{2+} mobilization in *wt-CASR*- and *CASR*^{T888M}-transfected cells. Responses were defined as the area under the 350/380 trace for the 'global' cluster, for the first 2 min following a change in Ca_o^{2+} concentration. *CASR*^{T888M} exhibited a leftward-shifted concentration-effect curve relative to *wt-CASR*, with a significantly reduced EC_{50} value (unpaired *t*-test; see inset; $^*P < 0.05$). (C) Anti-*CASR* immunoreactivity for *wt-CASR* and *CASR*^{T888M} showing 140 kDa core glycosylated protein, 160 kDa mature receptor, and a 240–310 kDa band representing *CASR* dimers (18).

Figure 3 Effect of $\text{CASR}^{\text{T888M}}$ mutation on phorbol ester-induced signal inhibition. (A) High Ca_o^{2+} concentration (4 mM) was used to elicit CASR-mediated Ca_i^{2+} mobilization resulting in oscillatory signaling for the wt-CASR and sustained Ca_i^{2+} signaling for $\text{CASR}^{\text{T888M}}$. Cotreatment with PMA (1 μM) abolished the responses of wt-CASR but had an impaired effect on $\text{CASR}^{\text{T888M}}$. Following removal of PMA and a brief recovery phase, cells regained or retained the responsiveness to high Ca_o^{2+} concentration (not shown). Median responses are shown and are representative of three separate coverslips. (B) The Ca_i^{2+} response to 4 mM Ca_o^{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. $\text{CASR}^{\text{T888M}}$ 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_o^{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_o^{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 $\text{CASR}^{\text{T888}}$ to certain non-phosphorylatable residues (e.g. alanine and valine) increased apparent Ca_o^{2+} sensitivity of the CASR, whereas phosphomimetic mutations (e.g. aspartate and glutamate) attenuated Ca^{2+} sensitivity (13, 14). Rather than altering Ca_o^{2+} sensitivity *per se* as an extracellular mutation might, mutation of $\text{CASR}^{\text{T888}}$ modulates receptor-induced signaling, preventing CASR-mediated Ca_i^{2+} mobilization when phosphorylated (as mimicked by $\text{CASR}^{\text{T888D}}$) but permitting sustained CASR-mediated Ca_i^{2+} mobilization when phosphorylation is prevented as in the case of $\text{CASR}^{\text{T888A}}$ *in vitro* (12–15) or $\text{CASR}^{\text{T888M}}$ *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 $\text{CASR}^{\text{T888}}$ residue exerts a critical function *in vivo*. Herein, we describe the clinical consequence of $\text{CASR}^{\text{T888}}$ mutation in humans. As predicted by the *in vitro* studies, we found that $\text{CASR}^{\text{T888M}}$

Figure 4 Effect of $\text{CASR}^{\text{T888M}}$ mutation on extracellular Ca^{2+} -induced ERK activation. (A) Representative western blots showing ERK-1/2 phosphorylation and total ERK-2 immunoreactivity in HEK-293 cells transfected transiently with either wt-CASR or $\text{CASR}^{\text{T888M}}$ following treatment for 10 min with different concentrations of Ca_o^{2+} up to 5 mM. (B) Quantification of the changes in ERK phosphorylation expressed as a percent of the maximal response in WT-CASR. $n = 6$ from two independent transfections. $**P < 0.01$ WT-CASR versus $\text{CASR}^{\text{T888M}}$ log EC_{50} values by paired t -test. (C) Similarly transfected cells were then stimulated with 4 mM Ca_o^{2+} for various time points up to 20 min, and ERK phosphorylation was quantified as before. Results are displayed here graphically (arbitrary densitometry units). $\text{CASR}^{\text{T888M}}$ elicited greater ERK phosphorylation after 1 min ($*P < 0.05$ paired t -test) with no differences observed at any other time point. Data comprise five replicates from two separate transfections: wt-CASR (black solid line) and $\text{CASR}^{\text{T888M}}$ (gray dashed line). (D) CASR immunoblot showing equivalent wt-CASR and $\text{CASR}^{\text{T888M}}$ immunoreactivity in the samples as before.

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_i^{2+} oscillations (29–32) in a PKC-sensitive manner, with PKC activation reducing the frequency of Ca_i^{2+} oscillations (30, 31). This suggests that CASR-mediated Ca_i^{2+} oscillations result from negative feedback provided by PKC-mediated phosphorylation at T888, producing dynamic phosphorylation and dephosphorylation of CASR^{T888} (31). In addition, we show here for the first time that downstream ERK phosphorylation is affected similar to Ca_i^{2+} mobilization by CASR^{T888} mutation, with CASR^{T888M} exhibiting greater Ca_o^{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^{T888} demonstrated PKC-mediated phosphorylation of the mature CASR in response to receptor activation, leading to feedback phosphorylation of CASR^{T888} (12). At lower levels of stimulation (i.e. 2 mM Ca_o^{2+}), CASR^{T888M} produced low frequency Ca_i^{2+} oscillations, whereas the relatively high concentration of 3 mM Ca_o^{2+} elicited mostly sustained responses (Figs 1 and 2) similar to that reported previously for CASR^{T888A} (8). We have also recently shown that the pattern of CASR-elicited Ca_i^{2+} mobilization is dependent on receptor-stimulated dephosphorylation of CASR^{T888}, as it permits either the next Ca_i^{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^{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^{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)₂D was initially elevated in the index case, but was normal in his father. The conversion of 25OHD to its active metabolite 1,25(OH)₂D is mediated by 1 α -hydroxylase, a crucial regulatory enzyme of predominantly renal origin. Hypocalcemia stimulates renal 1 α -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_o^{2+} concentration on 1 α -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_o^{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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References

- 1 Egbuna OI & Brown EM. Hypercalcaemic and hypocalcaemic conditions due to calcium-sensing receptor mutations. *Best Practice & Research. Clinical Rheumatology* 2008 **22** 129–148. (doi:10.1016/j.berh.2007.11.006)
- 2 Gunn IR & Gaffney D. Clinical and laboratory features of calcium-sensing receptor disorders: a systematic review. *Annals of Clinical Biochemistry* 2004 **41** 441–458. (doi:10.1258/0004563042466802)
- 3 Thakker RV. Diseases associated with the extracellular calcium-sensing receptor. *Cell Calcium* 2004 **35** 275–282. (doi:10.1016/j.ceca.2003.10.010)
- 4 Hendy GN, Guarnieri V & Canaff L. Chapter 3 calcium-sensing receptor and associated diseases. *Progress in Molecular Biology and Translational Science* 2009 **89C** 31–95. (doi:10.1016/S1877-1173(09)89003-0)
- 5 Hu J & Spiegel AM. Structure and function of the human calcium-sensing receptor: insights from natural and engineered mutations and allosteric modulators. *Journal of Cellular and Molecular Medicine* 2007 **11** 908–922. (doi:10.1111/j.1582-4934.2007.00096.x)
- 6 Lienhardt A, Bai M, Lagarde J, Rigaud M, Zhang Z, Jiang Y, Kottler M & Brown E. Activating mutations of the calcium-sensing receptor: management of hypocalcemia. *Journal of Clinical Endocrinology and Metabolism* 2001 **86** 5313–5323. (doi:10.1210/jc.86.11.5313)
- 7 Pearce SH, Williamson C, Kifor O, Bai M, Coulthard MG, Davies M, Lewis-Barned N, McCredie D, Powell H, Kendall-Taylor P, Brown EM & Thakker RV. A familial syndrome of hypocalcemia

- with hypercalciuria due to mutations in the calcium-sensing receptor. *New England Journal of Medicine* 1996 **335** 1115–1122. (doi:10.1056/NEJM199610103351505)
- 8 Mittelman SD, Hendy GN, Fefferman RA, Canaff L, Mosesova I, Cole DE, Burkett L & Geffner ME. A hypocalcemic child with a novel activating mutation of the calcium-sensing receptor gene: successful treatment with recombinant human parathyroid hormone. *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 2474–2479. (doi:10.1210/jc.2005-2605)
 - 9 Canaff L & Hendy GN. Human calcium-sensing receptor gene. Vitamin D response elements in promoters P1 and P2 confer transcriptional responsiveness to 1,25-dihydroxyvitamin D. *Journal of Biological Chemistry* 2002 **277** 30337–30350. (doi:10.1074/jbc.M201804200)
 - 10 Ward DT. Calcium receptor-mediated intracellular signalling. *Cell Calcium* 2004 **35** 217–228. (doi:10.1016/j.ceca.2003.10.017)
 - 11 Brennan SC & Conigrave AD. Regulation of cellular signal transduction pathways by the extracellular calcium-sensing receptor. *Current Pharmaceutical Biotechnology* 2009 **10** 270–281. (doi:10.2174/138920109787847484)
 - 12 Davies SL, Ozawa A, McCormick WD, Dvorak MM & Ward DT. Protein kinase C-mediated phosphorylation of the calcium-sensing receptor is stimulated by receptor activation and attenuated by calyculin-sensitive phosphatase activity. *Journal of Biological Chemistry* 2007 **282** 15048–15056. (doi:10.1074/jbc.M607469200)
 - 13 Bai M, Trivedi S, Lane CR, Yang Y, Quinn SJ & Brown EM. Protein kinase C phosphorylation of threonine at position 888 in Ca_v^{2+} -sensing receptor (CaR) inhibits coupling to Ca^{2+} store release. *Journal of Biological Chemistry* 1998 **273** 21267–21275. (doi:10.1074/jbc.273.33.21267)
 - 14 Jiang YF, Zhang Z, Kifor O, Lane CR, Quinn SJ & Bai M. Protein kinase C (PKC) phosphorylation of the Ca_v^{2+} -sensing receptor (CaR) modulates functional interaction of G proteins with the CaR cytoplasmic tail. *Journal of Biological Chemistry* 2002 **277** 50543–50549. (doi:10.1074/jbc.M205798200)
 - 15 McCormick WD, Atkinson-Dell R, Campion KL, Mun HC, Conigrave AD & Ward DT. Increased receptor stimulation elicits differential calcium-sensing receptor (T888) dephosphorylation. *Journal of Biological Chemistry* 2010 **285** 14170–14177. (doi:10.1074/jbc.M109.071084)
 - 16 Knox S, Harris J, Calton L & Wallace AM. A simple automated solid-phase extraction procedure for measurement of 25-hydroxyvitamin D₃ and D₂ by liquid chromatography–tandem mass spectrometry. *Annals of Clinical Biochemistry* 2009 **46** 226–230. (doi:10.1258/acb.2009.008206)
 - 17 Tan Y, Cardinal J, Franks A, Mun H, Lewis N, Harris L, Prins J & Conigrave A. Autosomal dominant hypocalcemia: a novel activating mutation (E604K) in the cysteine-rich domain of the calcium-sensing receptor. *Journal of Clinical Endocrinology and Metabolism* 2003 **88** 605–610. (doi:10.1210/jc.2002-020081)
 - 18 Ward DT, Brown EM & Harris HW. Disulfide bonds in the extracellular calcium-polyvalent cation-sensing receptor correlate with dimer formation and its response to divalent cations *in vitro*. *Journal of Biological Chemistry* 1998 **273** 14476–14483. (doi:10.1074/jbc.273.23.14476)
 - 19 Ward DT, McLarnon SJ & Riccardi D. Aminoglycosides increase intracellular calcium levels and ERK activity in proximal tubular OK cells expressing the extracellular calcium-sensing receptor. *Journal of the American Society of Nephrology* 2002 **13** 1481–1489. (doi:10.1097/01.ASN.0000015623.73739.B8)
 - 20 Bai M, Trivedi S & Brown EM. Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. *Journal of Biological Chemistry* 1998 **273** 23605–23610. (doi:10.1074/jbc.273.36.23605)
 - 21 Hough TA, Bogani D, Cheeseman MT, FAVOR J, Nesbit MA, Thakker RV & Lyon MF. Activating calcium-sensing receptor mutation in the mouse is associated with cataracts and ectopic calcification. *PNAS* 2004 **101** 13566–13571. (doi:10.1073/pnas.0405516101)
 - 22 Membreno L, Chen TH, Woodley S, Gagucas R & Shoback D. The effects of protein kinase-C agonists on parathyroid hormone release and intracellular free Ca^{2+} in bovine parathyroid cells. *Endocrinology* 1989 **124** 789–797. (doi:10.1210/endo-124-2-789)
 - 23 Nemeth EF, Wallace J & Scarpa A. Stimulus-secretion coupling in bovine parathyroid cells. Dissociation between secretion and net changes in cytosolic Ca^{2+} . *Journal of Biological Chemistry* 1986 **261** 2668–2674.
 - 24 Kobayashi N, Russell J, Lettieri D & Sherwood LM. Regulation of protein kinase C by extracellular calcium in bovine parathyroid cells. *PNAS* 1988 **85** 4857–4860. (doi:10.1073/pnas.85.13.4857)
 - 25 Morrissey JJ. Effect of phorbol myristate acetate on secretion of parathyroid hormone. *American Journal of Physiology. Endocrinology and Metabolism* 1988 **254** E63–E70.
 - 26 Nygren P, Gylfe E, Larsson R, Johansson H, Juhlin C, Klareskoq L, Akerstrom G & Rastad J. Modulation of the Ca^{2+} -sensing function of parathyroid cells *in vitro* and in hyperparathyroidism. *Biochimica et Biophysica Acta* 1988 **968** 253–260. (doi:10.1016/0167-4889(88)90014-6)
 - 27 Racke FK & Nemeth EF. Protein kinase C modulates hormone secretion regulated by extracellular polycations in bovine parathyroid cells. *Journal of Physiology* 1993 **468** 163–176.
 - 28 Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J & Hebert SC. Cloning and characterization of an extracellular $\text{Ca}(2+)$ -sensing receptor from bovine parathyroid. *Nature* 1993 **366** 575–580. (doi:10.1038/366575a0)
 - 29 Breitwieser GE. Calcium sensing receptors and calcium oscillations: calcium as a first messenger. *Current Topics in Developmental Biology* 2006 **73** 85–114. (doi:10.1016/S0070-2153(05)73003-9)
 - 30 Young SH & Rozengurt E. Amino acids and Ca^{2+} stimulate different patterns of Ca^{2+} oscillations through the Ca^{2+} -sensing receptor. *American Journal of Physiology. Cell Physiology* 2002 **282** C1414–C1422. (doi:10.1152/ajpcell.00432.2001)
 - 31 Young SH, Wu SV & Rozengurt E. Ca^{2+} -stimulated Ca^{2+} oscillations produced by the Ca^{2+} -sensing receptor require negative feedback by protein kinase C. *Journal of Biological Chemistry* 2002 **277** 46871–46876. (doi:10.1074/jbc.M207083200)
 - 32 Breitwieser GE & Gama L. Calcium-sensing receptor activation induces intracellular calcium oscillations. *American Journal of Physiology. Cell Physiology* 2001 **280** C1412–C1421.
 - 33 Bland R, Walker EA, Hughes SV, Stewart PM & Hewison M. Constitutive expression of 25-hydroxyvitamin D₃-1 α -hydroxylase in a transformed human proximal tubule cell line: evidence for direct regulation of vitamin D metabolism by calcium. *Endocrinology* 1999 **140** 2027–2034. (doi:10.1210/en.140.5.2027)
 - 34 Riccardi D, Hall AE, Chattopadhyay N, Xu JZ, Brown EM & Hebert SC. Localization of the extracellular Ca^{2+} /polyvalent cation-sensing protein in rat kidney. *American Journal of Physiology* 1998 **274** F611–F622.

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