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Novel activating mutation of human calcium-sensing receptor in a family with autosomal dominant hypocalcaemia

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ABSTRACT

Introduction: Autosomal dominant hypocalcaemia (ADH) is caused by activating mutations in the calcium sensing receptor gene (*CaR*) and characterised by mostly asymptomatic mild to moderate hypocalcaemia with low, inappropriately serum concentration of PTH.
Objective: The purpose of the present study was to biochemically and functionally characterise a novel mutation of *CaR*.
Patients: A female proband presenting with hypocalcaemia was diagnosed to have "idiopathic hypoparathyroidism" at the age of 10 with a history of muscle pain and cramps. Further examinations demonstrated hypocalcaemia in nine additional family members, affecting three generations.
Main outcome measure: P136L CaR mutation was predicted to cause gain of function of *CaR*.
Results: Affected family members showed relevant hypocalcaemia (mean ± SD; 1.91 ± 0.1mmol/l). Patient history included mild seizures and recurrent nephrolithiasis. Genetic analysis confirmed that hypocalcaemia cosegregated with a heterozygous mutation at codon 136 (CCC → CTC/Pro → Leu) in exon 3 of *CaR* confirming the diagnosis of ADH. For in vitro studies P136L mutant CaR was generated by site-directed mutagenesis and examined in transiently transfected HEK293 cells. Extracellular calcium stimulation of transiently transfected HEK293 cells showed significantly increased intracellular Ca^{2+} mobilisation and MAPK activity for mutant P136L CaR compared to wild type CaR.
Conclusions: The present study gives insight about a novel activating mutation of *CaR* and confirms that the novel P136L-CaR mutation is responsible for ADH in this family.

1. Introduction

CaR is a cell surface receptor for divalent cations. It is abundantly expressed in parathyroid glands, kidneys, and is also found in a wide variety of other tissues (Brown, 1997; Brown and MacLeod, 2001; Brown et al., 1998a, 1998b, 1999; Nemeth and Scarpa, 1987; Pearce et al., 1996a; Shoback et al., 1988). Stimulation of CaR plays a pivotal role in regulation of extracellular calcium homeostasis (Brown, 1997; Brown and MacLeod, 2001; Brown et al., 1998a, 1998b, 1999; Nemeth and Scarpa, 1987; Pearce et al., 1996a; Shoback et al., 1988). Furthermore, CaR regulation has been implicated in a variety of physiological and pathological processes in humans, such as hormone secretion, control of ion channels, or regulation of cell cycle events with proliferation, differentiation and apoptosis (Brown and MacLeod, 2001; Brown et al., 1994b; Kifor et al., 2001; Nagase et al., 2002; Tfelt-Hansen et al., 2003b, 2004). Autosomal dominant hypocalcaemia (ADH) is a familial syndrome characterised by the presence of inappropriately low parathyroid hormone levels with varying degrees of hypocalcaemia, hyperphosphatemia, and relative hypercalciuria (D'Souza-Li et al., 2002; Hendy et al., 2000; Okazaki et al., 1999; Pearce and Brown, 1996; Pearce et al., 1996b). ADH might be complicated by nephrolithiasis, nephrocalcinosis, and calcification of cerebral ganglia (Brown and MacLeod, 2001; D'Souza-Li et al., 2002; Hauache, 2001; Lienhardt et al., 2001; Pearce et al., 1996b; Watanabe et al., 1998; Yamamoto et al., 2000). Clinical presentation of ADH varies from asymptomatic hypocalcaemia to hypocalcaemic seizures, paraesthesias, sometimes with severe outcome manifesting with tetany or laryngospasm (D'Souza-Li et al., 2002; Tan et al., 2003). ADH has been previously demonstrated to be caused by activating mutations of *CaR*, which lead to suppression of PTH secretion by an increased sensitivity of CaR to extracellular calcium concentration. Genetic alterations of *CaR* were mapped to locus 3q13 on chromosome 3 (D'Souza-Li et al., 2002; Hauache, 2001; Hendy et al., 2000; Kifor et al., 2001; Lienhardt et al., 2001; Nagase et al., 2002; Okazaki et al., 1999; Watanabe et al., 1998).

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To date, more than 230 different disease-causing mutations of the CaSR have been reported (Hannan and Thakker, 2013). Furthermore about 95 different mutations have been identified in patients with ADH and sporadic hypoparathyroidism and are associated with the coding region for either the proximal extracellular domain (51%), for the transmembrane region (41%) or for the intracellular domain (8%) (Baron et al., 1996; D'Souza-Li et al., 2002; Hannan and Thakker, 2013; Hauache, 2001; Hendy et al., 2000; Lienhardt et al., 2001; Okazaki et al., 1999; Pearce, 2002; Pearce et al., 1996b; Pollak et al., 1994; Tan et al., 2003; Watanabe et al., 1998; Yamamoto et al., 2000). Depending on the potency of the activating mutation in CaR, patients can suffer not only from hypocalcaemia, hypomagnesemia and hypercalcuria, but also from renal loss of NaCl with polyuria, exhibiting symptoms similar to Bartter syndrome with secondary hyperaldosteronism and hypokalemia. To date, such severe courses of ADH were described for the following CaR mutations: E481K, A843E, C131W, L125P and K29E (Kinoshita et al., 2014; Lienhardt et al., 2000; Sato et al., 2002; Vargas-Poussou et al., 2002; Vezzoli et al., 2006; Watanabe et al., 2002). In the present study, a novel mutation of *CaR* (P136L) is reported in a family with ADH. Expression of P136L-CaR in HEK293 cells showed increased extracellular Ca^{2+}-dependent activation of intracellular Ca^{2+} mobilisation compared to wt-CaR. Moreover P136L-CaR showed a left-shift in the Ca^{2+}-dependent concentration curve of intracellular calcium release. Furthermore, calcium dependent stimulation of MAP-kinases ERK1/2, p38 and JNK were increased in HEK293 cells by expression of P136L-CaR compared to wt-CaR.

2. Materials and methods

2.1. Subjects

The family came to clinical attention because of symptomatic hypocalcaemia in individual II-7 [index patient, Fig. 1]. The index patient was diagnosed to have "idiopathic hypoparathyroidism" at age 10 after complaining of muscle pain and muscle cramps. Physical examination of the index patient revealed no abnormalities. CaR sequencing of the index patient demonstrated a novel mutation (P136L). Further examination demonstrated hypocalcaemia in nine additional family members, affecting three generations [Fig. 1, Table 1]. Biochemical analysis was prepared either at the time of first manifestation (individuals II-7 and III-3 to III-8) or at the time of genetic consultation (I-1, II-3, II-4). The results of serum examination and reference ranges were included in Table 2. Twenty-four-collected urine samples were analysed for adults and summarised in Table 3. Fractional excretion of Ca^{2+} was calculated as (urine Ca^{2+}*serum creatinine)/(serum Ca^{2+}*urine creatinine). Spot urine samples were collected from children and depicted in Table 3. Ca/Cr ratio was calculated as (urine Ca^{2+}/urine creatinine).

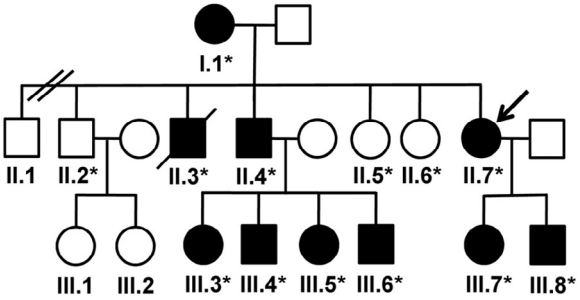

Fig. 1. Pedigrees of the three generation family with ADH, presenting with hypocalcaemia and low serum concentration of PTH caused by an activating *CaR* mutation. Index patient II-7 is indicated by *arrow*; all affected hypocalcaemic members (solid symbols) were demonstrated to have heterozygous P136L CaR mutation. Unaffected family members are characterised by open symbols. Individuals who provided samples for DNA sequencing are indicated by (*).

Table 1
Clinical findings in ten family members with ADH.

Pedigree no.	Age by manifestation	Symptoms	Blood pressure
I-1	64	BC, S	145/75
II-3	37	S, M	110/60
II-4	32	S	100/70
II-7	10	S,NC,NL	110/80
III-3	11	MP	110/60
III-4	7	NO	100/50
III-5	5	NO	90/60
III-6	3	M, MP, S,	85/55
III-7	1	NO	ND
III-8	2	NO	ND
Mean ± SE	17 ± 20		

BC, basal ganglia calcification; M, myoclonia; MP, muscle pain; NA, not available; NC, nephrocalcinosis; NL, nephrolithiasis; NO, no clinical symptoms; S, seizures; RC, renal calcification; T, tremulousness.
Six of 10 family members were symptomatic, mostly the third generation did show no symptoms.
Blood pressure was normal for resp. age group.

2.2. Direct sequence analysis of CaR exons

Genomic DNA was obtained from peripheral blood leukocytes and isolated using standard methods (Tan et al., 2003). Protein-coding exons 2-7 of the *CaR* were amplified by PCR, as described previously (D'Souza-Li et al., 2002; Hendy et al., 2003; Janicic et al., 1995; Lienhardt et al., 2000; Pearce et al., 1995; Pollak et al., 1993; Zajickova et al., 2007). Amplification products were purified and nucleotide sequences of both strands were determined by direct sequencing and compared to reference DNA sequence.

2.3. DNA amplification, site-directed mutagenesis and sequence analysis

For studying protein structure–function relationships and gene expression, site-directed mutagenesis was used. We first cloned the wt-*CaR* in XL10-Gold® Ultracompetent Cells (STRATAGENE, La Jolla, CA). Plasmid pcDNA3.1, containing the wt-*CaR* (GenBank accession no.U20759), a the generous gift of Mei Bai and Ed Brown (Endocrine-Hypertension Division, Brigham and Women's Hospital, Boston, MA). To create mutant receptor the Quick-change-XL-II-site directed mutagenesis kit was used (STRATAGENE, La Jolla, CA). To yield the mutation in which the CCC (Proline) residue at position 136 was substituted to CTC (Leucine), in Exon 3 in *CaR*, the mutagenic oligonucleotides were used: forward primer, 5′CTGCTCAGAGCACATTCCCTCTACGATTGCTGTG 3′; and reverse primer, 5′CACAGCAATCGTAGAGGGAATGTGCTCTGAGCAG 3′ for P136L mutation (Eurofins MWG Operon, Ebersberg, Germany). The plasmids were isolated using S.N.A.P.™ MiniPrep Kit (Invitrogen) when the suspension culture reached up to $OD_{600} > 1.0$, measured using Spectrophotometer (Eppendorf). PCR products were verified by direct double stranded sequencing (QIAGEN, Hilden, Germany). The verification of the insert sequence confirmed the desired mutation in selected clones. The plasmid probes of concentration of about 30 μg/ml were used for further experiments.

2.4. Cell culture and transient transfection

It has been previously shown that HEK293 cells do not express endogenous CaR (Galitzer et al., 2009). For functional characterisation of P136L mutant receptor, *CaR* wild-type and P136L-*CaR* were transiently transfected into HEK 293 cells (Bai et al., 1996; Lienhardt et al., 2001; Okazaki et al., 1999). Cells were cultured in DMEM