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A missense mutation in the seventh transmembrane domain constitutively activates the human Ca^{2+} receptor

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Abstract A missense mutation, A843E, in the seventh transmembrane domain of the human Ca^{2+} receptor, identified in a subject with autosomal dominant hypocalcemia, was found to cause a constitutive activation while at the same time lowering the maximal response of the receptor to Ca^{2+}. A truncated human Ca^{2+} receptor lacking the majority of the N-terminal extracellular domain failed to respond to Ca^{2+} despite an excellent cell surface expression. The A843E mutant version of this truncated receptor showed constitutive activation. These results identify A843 as a critical residue for maintaining the inactive conformation of the human Ca^{2+} receptor. Substitution of glutamate, but not lysine or valine, for alanine 843 leads to activation of the human Ca^{2+} receptor in a manner that no longer depends upon Ca^{2+} binding to the extracellular domain.

Key words: G protein-coupled receptor; Constitutive activation; Calcium-sensing receptor

1. Introduction

Activating mutations of G protein-coupled receptors (GPCR) have been identified as the cause of several human diseases [1]. Functional expression studies of such mutant receptors have in general shown that they are constitutively activated, i.e. they activate G protein in an agonist-independent manner (for example [2]). Activating mutations have also been identified in the human Ca^{2+} receptor (hCaR) in a disorder termed autosomal dominant hypocalcemia (ADH) [3]. The CaR is a member of the GPCR gene superfamily that is expressed at particularly high levels in parathyroid and kidney where it serves to regulate the extracellular Ca^{2+} metabolism [4]. Extracellular Ca^{2+} directly activates the CaR causing inhibition of parathyroid hormone (PTH) secretion from the parathyroids and a decreased renal Ca^{2+} reabsorption. Germline heterozygous, activating missense mutations of the hCaR, cause inhibition of the PTH secretion and a decreased renal Ca^{2+} reabsorption at an inappropriately low serum Ca^{2+} concentration, leading to the hypocalcemia, relative hypercalciuria and an inappropriately low serum PTH that characterizes subjects with ADH [5].

Functional expression of hCaRs with mutations identified in subjects with ADH to date has shown that they are not, in fact, constitutively activated. Activities of mutant and wild-type receptors do not differ at a very low Ca^{2+} concentration, but the mutant receptors show an enhanced sensitivity to Ca^{2+} with an EC_{50} shifted leftward by a factor of almost two [3,6–8]. Most mutations identified in subjects with ADH are located in the large, ~600 residue N-terminal extracellular domain (ECD) of the hCaR where they might be expected to influence the sensitivity to Ca^{2+} activation, but even the few mutations identified within the seven transmembrane portion of the CaR in subjects with ADH have shown a pattern of increased sensitivity to Ca^{2+} rather than constitutive activation [7,9].

During the course of our studies on expression of mutant CaRs, we found that a mutation, alanine 843 to glutamate (A843E), identified in the seventh transmembrane domain of the hCaR in a subject with hypocalcemia and hypercalciuria [10] causes a true constitutive activation. Substitution of A843 by amino acids other than glutamate did not lead to constitutive activation. By constructing a form of the hCaR that lacked the ECD but still was expressed at the cell surface, we were able to show that constitutive activation caused by the A843E mutation does not depend on an intact ECD. These studies offer a new insight into the mechanism of activation of the hCaR.

2. Materials and methods

2.1. Site-directed mutagenesis

The entire encoding region of the hCaR [11] was subcloned in pCR3.1 (Invitrogen, San Diego, CA, USA). Site-directed mutagenesis was carried out by using the QuickChange site-mutagenesis kit (Stratagene, La Jolla, CA, USA). The pairs of oligonucleotide primers used for each mutation are available on request from the authors. All mutations were confirmed by sequencing using ABI prism dRhodamine terminator cycle sequencing ready reaction kit and ABI prism-377 sequencer (Applied Biosystems, Foster City, CA, USA).

2.2. Construction of N-terminal-truncated hCaR with a rhodopsin epitope tag (Rho-C-hCaR)

A polymerase chain reaction (PCR) was used to add 20 amino acid residues (MNGTEGPNFYVPFSNKTGVV) corresponding to the N-terminus of bovine rhodopsin to the hCaR at residue 599. The sense primer used was a 100 base oligonucleotide corresponding to the nucleotide sequence of the N-terminal 20 amino acids of bovine rhodopsin and the nucleotide positions 1797–1832 of hCaR cDNA: 5′-CAGCCATGAACGGGACCGAGGGCCCAAACTTCTACGTGC-CTTTCTCCAACAAGACGGGCGTGGTGGCCAAGGAGATCG-AGTTTCTGTCGTGGACGGAGCC-3′. The antisense primer used was a 46 base oligonucleotide corresponding to the nucleotide positions 2667–2709 of hCaR cDNA and a stop codon, 5′-CCTAT-CCAAGGCTGCTGGACCGCTTGCGGGAGACGTTGCTGCGG-CG-3′. The PCR was done by using the Advantage cDNA PCR kit (Clontech, Palo Alto, CA, USA). The PCR product was subcloned to the PCDNA3.1 vector by using the Eukaryotic TA cloning kit (Invi-

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Abbreviations: CaR, Ca^{2+} receptor; hCaR, human Ca^{2+} receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ADH, autosomal dominant hypocalcemia; PI, phosphoinositide; GPCR, G protein-coupled receptor; ECD, extracellular domain; PCR, polymerase chain reaction; TM, transmembrane domain; mGluR, metabotropic glutamate receptor

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trogen, San Diego, CA, USA). The entire sequence of the PCR product was confirmed by using the ABI prism dRhodamine terminator cycle sequencing ready reaction kit and an ABI prism-377 sequencer (Applied Biosystems, Forster City, CA, USA). The truncated mutant hCaR, designated Rho-C-hCaR, includes 20 amino acids of the N-terminus of rhodopsin and the amino acid residues 599–903 of hCaR.

2.3. Transient transfection of wild-type and mutant hCaRs in HEK-293 cells

Transfections were performed as previously described [11]. In brief, hCaR cDNAs for transfection were prepared by the QIAGEN Maxi plasmid DNA preparation kit (QIAGEN, Chatsworth, CA, USA). Lipofectamine (Life Technologies, Gaithersburg, MD, USA) was employed as the DNA carrier for transfection. The HEK-293 cells used for transfection were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (BioFluids, Rockville, MD, USA), 1% glutamine and 1% penicillin/streptomycin. The DNA-liposome complex was prepared by mixing DNA and Lipofectamine in DMEM and incubating the mixture for 30 min at room temperature. The DNA-Lipofectamine complex was further diluted in serum-free DMEM and added to 90% confluent HEK-293 cells. After 5 h of incubation, an equal volume of regular medium with 20% FBS was added and the medium was replaced 24 h after transfection with fresh medium.

2.4. Phosphoinositide hydrolysis assay

The assay was performed as previously described [12,13]. Briefly, HEK-293 cells grown in supplemented DMEM were plated in 24 well culture plates at 80–90% confluency. 24 h after being transfected with wild-type and mutant hCaR, cells were incubated with 3.0 μCi/ml [^{3}H]myoinositol (New England Nuclear, Boston, MA, USA) in full medium (DMEM+10% FBS+1% glutamine+1% and 1% penicillin/streptomycin) for 16–24 h, followed by 30 min preincubation with PI buffer (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 20 mM LiCl in 25 mM PIPES buffer, pH 7.2). Cells were stimulated with various extracellular Ca^{2+} concentrations in PI buffer for 30 min. The reaction was terminated by the addition of 1 ml acid-methanol (167 μl HCl in 120 ml methanol). Total [^{3}H]inositol phosphates were extracted and separated on Dowex AG1-X8 columns as described [12,13].

2.5. An intact cell enzyme-linked immunoassay (ELISA) to determine the cell surface expression of transfected receptors

Transfected HEK-293 cells in six well plates were detached with 1 mM EDTA in PBS containing 0.5% bovine serum albumin. To measure the cell surface expression of wild-type and mutant CaR with an intact ECD, cells were incubated in DMEM for 1 h on ice with 1 μg/ml monoclonal antibody 7F8 raised against the purified ECD of hCaR as previously described [11,14]. The cell surface expression was calculated as the percentage OD compared with the value of wild-type hCaR (set as 100%) after subtracting the OD value of cells transfected with vector alone. To measure the cell surface expression of receptors with a rhodopsin epitope tag, a monoclonal antibody against the N-terminus of rhodopsin, B6-30N [15], was used instead of 7F8.

3. Results

3.1. Analysis of the mutant hCaR signal transduction using a phosphoinositide hydrolysis assay

The function of wild-type and mutant hCaRs expressed in transfected HEK-293 cells was assessed by measuring the PI hydrolysis in response to increasing concentrations of Ca^{2+}. The response of two different missense mutants identified in subjects with ADH, alanine 116 to threonine (A116T) [16] and A843E [10], was compared in this assay with the wild-type hCaR (Fig. 1). The A116T mutant, like other activating hCaR mutations studied to date [3,6,7,9], shows no significant difference from the wild-type receptor at very low Ca^{2+} concentrations, but its EC_{50} is shifted leftward ~2-fold. The PI response of the A843E mutant, in contrast, even in the absence of extracellular Ca^{2+}, corresponds to 50–60% of the maximum response of the wild-type hCaR. The A843E mutant, however, does not reach the same level of activation as the wild-type receptor even at very high Ca^{2+} concentrations. The A843E mutant Ca^{2+}-independent activation of PI hydrolysis was observed over a range of transfected cDNA from 0.25 to 6 μg/ml. The PI assay in the absence of Ca^{2+} of cells transfected over this range with either wild-type or A116T mutant hCaR cDNAs showed a constant low level of activity. In contrast, transfection with A843E mutant cDNA increased

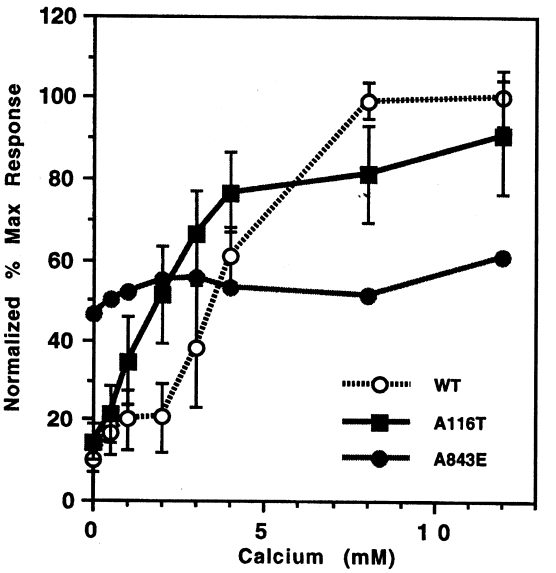

Fig. 1. Concentration-dependence for the Ca^{2+} stimulation of phosphoinositide hydrolysis in transiently transfected HEK-293 cells expressing wild-type and activating mutant (A116T and A843E) hCaR cDNAs. Each data point is the mean value of triplicate determinations from six independent experiments. All responses are normalized to the maximum response of the wild-type hCaR. The S.E.M. is marked with a vertical bar through each point.

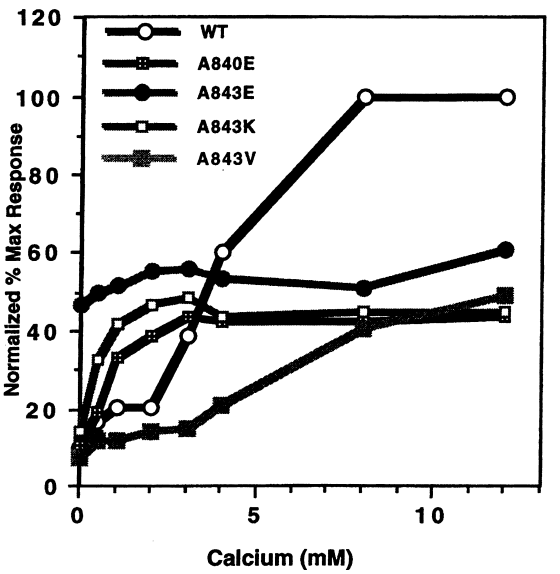

Fig. 2. Concentration-dependence for the Ca^{2+} stimulation of phosphoinositide hydrolysis in transiently transfected HEK-293 cells expressing the wild-type, A843(E, K or V) and A840E mutant hCaR cDNAs. The data are expressed as described in the legend to Fig. 1 except that the S.E.M. which was $< 10\%$ is not shown.

the activity in this assay compared with the wild-type at every concentration of DNA used, with a peak activity at 1 μg/ml DNA (data not shown).

To determine whether constitutive activation caused by the A843E mutation is due to substitution of A843 or specifically requires the substitution by glutamate, we created several addition mutants, substituting A843 with either positively charged lysine (A843K) or uncharged valine (A843V). We also made another mutant in which a neighboring alanine in the seventh transmembrane domain is substituted by glutamate (A840E). Unlike A843E, none of these mutants caused a constitutive activation, although like A843E, the maximal activation of all these mutants was only 50–60% of that of the wild-type (Fig. 2). The PI response of A840E and A843K mutants demonstrated a leftward shift compared to wild-type, whereas the A843V mutant showed a rightward shift compared to wild-type.

3.2. Cell surface expression assessed by an intact cell ELISA

In order to determine if the differences in activity observed for the mutant hCaRs were due to differences in cell surface expression, we measured the cell surface expression level with an ELISA using a monoclonal antibody raised against the purified ECD of hCaR. As illustrated in Fig. 3, mutant hCaRs (A116T, A840E, A843K, A843V) were all expressed at levels close to that of the wild-type. Failure of the maximum PI response of mutant hCaRs (A840E, A843K, A843V) to reach wild-type levels cannot therefore be ascribed to a reduced cell surface expression. As for the constitutively activating mutant A843E, its average cell surface expression level is significantly lower, corresponding to 85% of the wild-type expression. Thus, the increased cell surface expression cannot account for the constitutive activation observed for the A843E mutant.

3.3. Expression of truncated hCaRs lacking the ECD

We hypothesized that the ability of the A843E mutation to constitutively activate the hCaR was not dependent on an intact ECD. To test this hypothesis, we sought to construct a truncated form of the hCaR lacking the ECD. We have shown previously that truncation of the long intracellular C-terminus of the hCaR at residue 903 does not impair receptor activation or G protein coupling and may enhance the cell surface expression [11]. We therefore combined the C-terminal truncation at residue 903 with truncation of the ECD proximal to the first transmembrane domain to generate mutants lacking the ECD. We found that such mutants were not expressed at the cell surface even if we included the hCaR signal sequence at the N-terminus (data not shown). To circumvent this problem, we created a mutant (rho-C-hCaR) with the C-terminal residue 903 truncation combined with a N-terminus consisting of the first 20 residues of bovine rhodopsin followed by the remainder of the hCaR beginning with residue 599. The N-terminal rhodopsin sequence contains an epitope for the rhodopsin monoclonal antibody, B6-30N, providing a convenient means of monitoring the cell surface expression of rho-C-CaR. Immunoblots showed an excellent expression of rho-C-CaR (data not shown). We next generated a form of rho-C-hCaR containing the A843E mutation and compared the PI hydrolysis response to Ca^{2+} of cells transfected with rho-C-hCaR, rho-C-hCaR(A843E) and wild-type hCaR cDNAs. Rho-C-hCaR showed no response to Ca^{2+}, whereas the rho-C-hCaR(A843E) mutant showed constitutive activation at a level only slightly lower than that of the full length A843E hCaR mutant (Fig. 4). Failure of the rho-C-hCaR mutant to respond to Ca^{2+} was not due to a lack of cell surface expression, since the intact cell ELISA using the rhodopsin monoclonal antibody showed that rho-C-hCaR and rho-C-hCaR(A843) were expressed at equivalent levels (OD values between four and five times that of control cells transfected with the vector only).

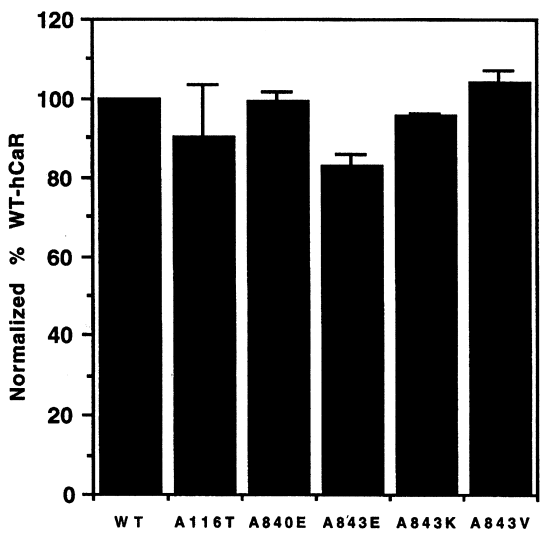

Fig. 3. Intact cell ELISA for the quantitation of cell surface expression of wild-type and mutant hCaRs. The data are normalized as a percentage of the value of the wild-type hCaR (set as 100%) using HEK-293 cells transfected with vector alone as the negative control. Each value represents the mean+S.E.M. of duplicate measurements from four independent experiments.

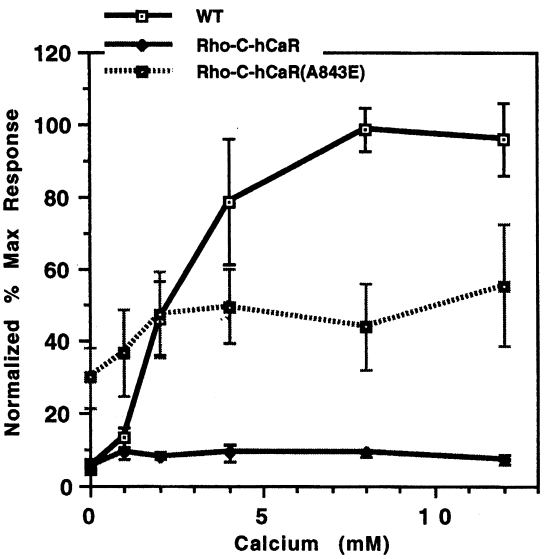

Fig. 4. Concentration-dependence for the Ca^{2+} stimulation of phosphoinositide hydrolysis in transiently transfected HEK-293 cells expressing the wild-type, rho-C-hCaR and rho-C-hCaR(A843E) cDNAs. Data are expressed as in the legend to Fig. 1.

4. Discussion

A current model for GPCR activation postulates a dynamic

equilibrium between a conformation favoring G protein coupling (R*) and one that does not favor G protein coupling [17]. In the latter conformation, inhibitory constraints, including possible interactions between key residues in several of the transmembrane helices, prevent the spontaneous formation of R*. In this model, agonist binding stabilizes the R* conformation by somehow relieving the inhibitory constraints. For GPCR in the rhodopsin subfamily in which agonists bind directly within the seven transmembrane barrel, one can easily imagine agonist-promoted alterations in interhelical interactions that change the configuration of intracellular loops to promote G protein coupling. For GPCRs such as the glycoprotein hormone receptors and the CaR/mGluR subfamily which have large ECDs that are believed to be the site of agonist binding, how agonist binding leads to GPCR activation is far less clear.

Many naturally occurring, activating missense mutations have been identified in various GPCR in a number of diseases [1]. These missense mutations have provided an unique insight into the mechanism of GPCR activation. Upon expression of such mutated receptors, these have generally been shown to cause an agonist-independent, constitutive activation leading to the suggestion that mutation of the involved residues disrupts the normal inhibitory constraints and allows the formation of R*. In contrast, activating CaR mutations have shown an increased sensitivity to Ca^{2+} rather than true constitutive activation [3,6–8]. Of the 14 activating CaR mutations identified to date, nine are located in the ECD, a region suggested to be important for Ca^{2+} binding [18]. Missense mutations of the ECD may therefore lead to an inappropriate CaR activation by increasing the affinity for Ca^{2+}. However, even mutations in the transmembrane domains of the CaR (F788C and L773R in TM5, F806 in TM6) or in the first extracellular loop (Q681H) have shown an enhanced sensitivity to Ca^{2+} rather than constitutive activation when expressed [7,10,16].

The A843E mutation in TM7 functionally characterized in this paper is the first example of a CaR mutation causing a constitutive activation rather than an enhanced Ca^{2+} sensitivity. The A843 residue is conserved in human [19], bovine [18], rat [20], chicken [21] and fugu [22] CaRs, as well as in the closely related putative pheromone receptor, mv2R2 [23], consistent with its functional importance. The comparable residue is glycine, however, in the less closely related metabotropic glutamate receptors (mGluR) [19]. We found that substitution of glutamate for alanine 843 was required for constitutive activation. Neither substitution of lysine or valine for alanine 843 nor glutamate substitution for the nearby and also highly conserved alanine 840 led to a constitutive activation. Interestingly, all three of these mutations, like A843E, lowered the maximal Ca^{2+} activation compared to wild-type CaR. Some constitutively activating mutations of other GPCR have also been associated with a reduced level of maximal agonist activation, e.g. the L457R mutation identified in TM3 of the LH receptor in a subject with gonadotropin-independent precocious puberty [24].

The unique effects seen with the A843E mutation of the CaR lead us to suggest, in keeping with the prevalent model of GPCR activation, that this mutation, without Ca^{2+} binding, disrupts the inhibitory constraints that prevent G protein coupling. It is possible that the mutation leads to changes in side chain packing in TM7 or in contacts with residues in other TM helices. Such changes could alter the configuration of one or more intracellular loops in such a way as to favor G protein coupling. The inability to achieve a maximal activation to wild-type levels may signify that the mutation, while promoting a conformation favouring G protein coupling, may at the same time prevent the CaR from assuming the specific conformation most favorable for G protein coupling. Unfortunately, all of these possibilities must remain speculative in the absence of a rigorously defined structure for the CaR.

We were able, however, to test our hypothesis that the A843E mutation activates the CaR by promoting G protein coupling without requiring Ca^{2+} binding to the ECD. By attaching an N-terminal 20 amino acid segment of rhodopsin to a CaR without the majority of the ECD (rho-C-hCar), we were able to achieve high levels of cell surface expression. The observation that rho-C-hCaR fails to respond to Ca^{2+} is consistent with the postulated role of the ECD in Ca^{2+} binding [18]. The fact that rho-C-hCaR is not constitutively activated argues against a model of receptor activation in which the ECD exerts a tonic inhibitory effect through interaction with the TM7 portion of the receptor, an inhibitory effect normally relieved by agonist binding to the ECD. Such a model has been proposed for the TSH receptor, based in part upon the ability of low concentrations of trypsin or of certain ECD mutations to activate that receptor [25]. A direct test of this model has not been done, since up to now a truncated TSH receptor lacking the ECD has not been successfully expressed in a form that reaches the plasma membrane.

The A843E mutation, when expressed in the context of rho-C-hCaR, still caused constitutive activation supporting the idea that this mutation alters the conformation of the TM7 portion of the receptor in a manner independent of both Ca^{2+} and the ECD. This still leaves open the question of how Ca^{2+} binding and the ECD normally lead to receptor activation. Based on the results seen with rho-C-hCaR, and by analogy with the function of bacterial periplasmic binding proteins to which the mGluR1 [26] and CaR [27] ECDs have been noted to be homologous, we currently favor a model in which Ca^{2+} binding causes a change in the conformation of the ECD that promotes an interaction with the TM7 portion of the CaR. This interaction alters helical interactions in a way that leads to G protein coupling. Such a model would predict that there may be other mutations of the TM7 portion of the receptor such as A843E that will cause constitutive activation and that there may also be residues in the postulated interface between ECD and TM7 portions of the receptor which when mutated will prevent receptor activation. Further studies to test this model are underway.

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