Mutational analysis of the cysteines in the extracellular domain of the human Ca^{2+} receptor: effects on cell surface expression, dimerization and signal transduction

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Abstract Mammalian calcium receptors (CaRs) share with the metabotropic glutamate receptors (mGluRs) the relative positions of 16 cysteine residues in the amino-terminal extracellular domain. To investigate the role of these cysteines, a series of mutants in the extracellular domain of the human CaR was prepared in which each of these 16 cysteine residues and three others not conserved in the mGluRs were replaced by serines. Wild-type and mutant CaR cDNAs were expressed in HEK-293 cells, and evaluated for expression and response to extracellular calcium. Mutation of three non-conserved cysteines and of two conserved cysteines produced proteins with near wild-type phenotype. In contrast, mutation of the other conserved cysteines gave proteins that showed drastic reduction in cell surface expression and/or failed to respond to calcium. We identified 14 cysteines essential for proper trafficking and function of the receptor, two of which may be involved in formation of a disulfide-linked dimer.

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Key words: G protein-coupled receptor; Disulfide; Human calcium receptor

1. Introduction

The calcium receptor (CaR) is a G protein-coupled receptor that plays a central role in extracellular calcium homeostasis by mediating the effect of extracellular Ca^{2+} on parathyroid hormone secretion and on renal calcium excretion [1]. In addition to the parathyroids and kidney, the CaR is expressed in brain, intestine, skin, and other organs where it may mediate the effects of extracellular Ca^{2+} on a variety of other cellular functions such as ion channel activity and proliferation [2]. While the CaR shares with other members of the superfamily of G protein-coupled receptors a characteristic seven transmembrane-domain structure, its sequence and topography are otherwise unique. In particular, the CaR possesses a large (~600 amino acid), amino-terminal extracellular domain (ECD) which was noted [3,4] to be similar in length and to a limited extent in amino acid sequence to the ECD of the metabotropic glutamate receptors (mGluRs), themselves a unique subfamily of G protein-coupled receptors [5,6]. A distinctive feature of the CaR ECD is the presence of 19 cysteine residues, nine clustered between residues 542 and 598 of the human CaR (hCaR) sequence in what has been termed a cysteine-rich region, and the remaining ten scattered within the first 500 residues of the ECD.

All 19 of these cysteines are conserved in mammalian CaRs including human [4], bovine [3], rat [7,8] and rabbit [9], and all but cysteine 482 (hCaR sequence) are conserved in the chicken CaR [10]. Sixteen of the 19 cysteines (all except residues 129, 437, and 482 of the hCaR) are conserved in the identical positions in the amino-terminal ECDs of the seven members of the mGluR subfamily of G protein-coupled receptors [6]. The conservation of CaR ECD cysteines among different species, and between the CaR and mGluRs, suggests an important role for these cysteines in the structure and perhaps function of these receptors. Although these conserved ECD cysteines have been noted by several groups who have speculated on their potential significance [3,5], and there is evidence that both mGluRs and the CaR form disulfide-linked dimers [11–13], experimental studies to define the role of specific ECD cysteines have not been done for either the CaR or mGluRs. As an initial step in defining the role of the ECD cysteines in CaR structure and function, we mutated each of the individual 19 ECD cysteines to serine, and assessed the ability of the mutant receptors to be expressed, to traffic to the cell surface, to form dimers, and to activate phosphoinositide (PI) hydrolysis in response to extracellular Ca^{2+}.

2. Materials and methods

2.1. Site-directed mutagenesis of the hCaR

Site-directed mutagenesis was performed using QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) on the hCaR cDNA amplified by polymerase chain reaction from a Pituitary Marathon cDNA library (Promega, Madison, WI, USA) and cloned into pCR 3.1 (Invitrogen, San Diego, CA, USA) mammalian expression vector as previously described [14]. The complementary primer pairs used are available on request from the authors. The presence of cysteine to serine point mutations and the absence of other mutations were confirmed by automated DNA sequencing using ABI prizm-377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.2. Transient expression of wild-type and mutant hCaR receptors in HEK-293 cells

Receptor cDNAs in pCR 3.1 were prepared by Qiagen maxi-plasmid DNA preparation kit (Qiagen, Chatsworth, CA, USA) and were transfected into HEK-293 cells using lipofectamine (Life Technologies, Gaithersburg, MD, USA) as previously described [14], using 0.5 μg of DNA per well on 24-well plates (for phosphoinositide (PI) hydrolysis assay), in 6-well plates using 1 μg of DNA (for intact cell ELISA), and in 75-mm flasks using 10 μg of DNA (for immunoblot analysis). Membrane protein extraction for immunoblotting, intact cell ELISA and PI hydrolysis assay were performed 48 h after transfection, as previously described [14].

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Abbreviations: CaR, calcium receptor; mGluR, metabotropic glutamate receptor; ECD, extracellular domain; PI, phosphoinositide; ELISA, enzyme-linked immunosorbent assay

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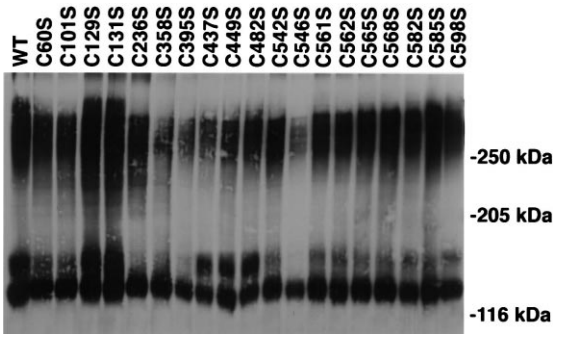

Fig. 1. Immunoblot showing the expression pattern of wild-type hCaR and cysteine→serine mutants transiently expressed in HEK-293 cells. Crude membrane extracts were obtained from HEK-293 cells that were transiently transfected with wild-type hCaR and different cysteine→serine mutant plasmid cDNAs as described in Section 2. 60 μg of membrane protein from cells transfected with each receptor were loaded in each lane (labeled at top; WT = wild type), and resolved on 5–15% gradient gel by SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed with 0.1 μg/ml monoclonal anti-hCaR antibody ADD raised against a synthetic peptide corresponding to residues 214–235 of hCaR [15]. The positions of molecular size standards are indicated at the right.

3. Results

3.1. Immunoblot analysis of the expression of hCaR cysteine→serine mutants

Wild-type and 19 different cysteine→serine mutant hCaR cDNAs were transiently transfected in HEK-293 cells, and expression assessed by immunoblot. The peptide used to generate the ADD monoclonal antibody used for immunoblot includes none of the 19 ECD cysteines. Also, the ADD monoclonal antibody has been shown to bind strongly to the denatured CaR and CaR ECD [15]. Thus, as long as some receptor protein was expressed, we expected the ADD monoclonal to recognize each of the cysteine mutant CaRs on immunoblots, even if the mutation caused misfolding and/or impaired protein processing and trafficking. Membrane preparations of cells transfected with vector containing the wild-type hCaR cDNA showed a characteristic pattern (Fig. 1) consisting of a doublet of ~150 kDa and ~130 kDa, representing fully glycosylated and incompletely processed, high mannose forms of the CaR, respectively, and a higher molecular weight smear which may represent dimeric forms of the receptor as previously reported [13,14,16,17]. Immunoblots of membranes from HEK-293 cells transfected with vector alone showed no immunoreactivity [15,17]. The cysteine mutants fell into two broad classes based on the immunoblotting shown in Fig. 1. Expression of C129S, C131S, C437S, C449S and C482S mutants gave products similar to those obtained with the wild type with prominent ~150-kDa and ~130-kDa bands. All the other cysteine to serine mutants showed markedly decreased immunostaining of the 150-kDa band, but retained strong immunostaining of the 130-kDa band.

3.2. Intact cell enzyme-linked immunoassay to assess cell surface expression level of hCaR cysteine mutants

To determine the level of cell surface expression of the mutant receptors, we took advantage of an intact cell ELISA using monoclonal antibodies raised against the purified ECD of the hCaR, 7F8 and 7B10. Both of these antibodies bind to the native hCaR expressed in HEK 293 cells (P.K. Goldsmith and A.M. Spiegel, manuscript in preparation). As illustrated in Fig. 2, the optical densities (measured with antibody 7F8) indicated that the C129S, C131S and C482S mutant receptors were expressed at a level close to that of the wild-type receptor (88%, 97% and 89%, respectively). OD values for C437S and C449S mutants were significantly lower (64% and 65% of wild type, respectively). The C60S and C598S mutants showed a cell surface expression nearly half of that of the wild-type receptor. Very low levels of cell surface expression (from not detectable to 28%) were observed in the other mutants. Intact cell ELISA using another monoclonal antibody raised against the purified ECD, 7B10, gave similar results (data not shown).

3.3. Analysis of signal transduction by cysteine mutant receptors using phosphoinositide hydrolysis assay

Since the CaR responds to increases in $[Ca^{2+}]_o$ by activating Gq family proteins to stimulate PLC-β activity with resultant PI hydrolysis, we analyzed the effect of increasing $[Ca^{2+}]_o$ on the accumulation of inositol phosphates in transfected HEK-293 cells to measure signal transduction by expressed wild-type and mutant CaRs. Three cysteine mutants, C129S, C131S and C482S, showed Ca^{2+} response similar to that of the wild type-receptor (Fig. 3). Two other cysteine mutants, C437S and C449S, showed a rightward shift of the Ca^{2+} concentration-response curve with a decreased maximal response (Fig. 3). In contrast, all other cysteine mutant receptors showed no significant PI hydrolysis response to $[Ca^{2+}]_o$ even up to 20 mM (data not shown), a result equivalent to that seen with cells transfected with vector alone (Fig. 3).

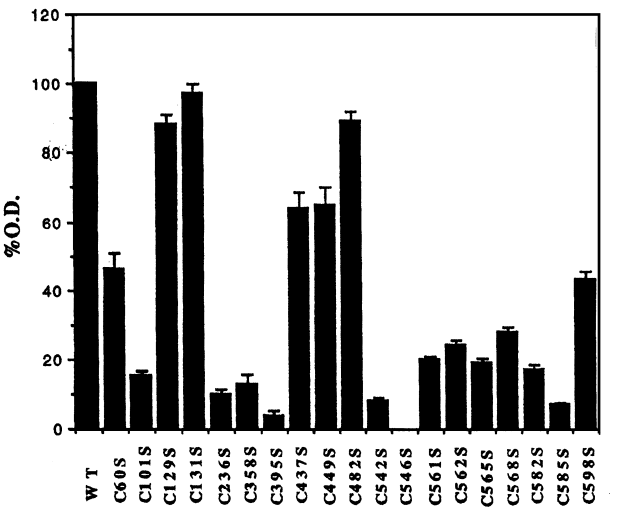

Fig. 2. Intact cell enzyme-linked immunoassay for quantitation of cell surface expression of wild-type and cysteine→serine mutant hCaRs. HEK-293 cells in 6-well plates were transfected with 1 μg/well of wild-type or cysteine→serine mutant hCaR cDNAs. Cells in suspension were incubated with 1 μg/ml 7F8 or 7B10 monoclonal hCaR antibody raised against the purified hCaR ECD and with peroxidase-conjugated anti-mouse immunoglobin secondary antibody as described in Section 2. Peroxidase substrate was added and OD was measured at 405 nm. The OD readings are expressed as a per cent of the value for the wild-type hCaR (set at 100%) using HEK-293 cells transfected with vector alone as the negative control. Each point represents the mean ± S.E.M. of duplicate determinations from three independent experiments.

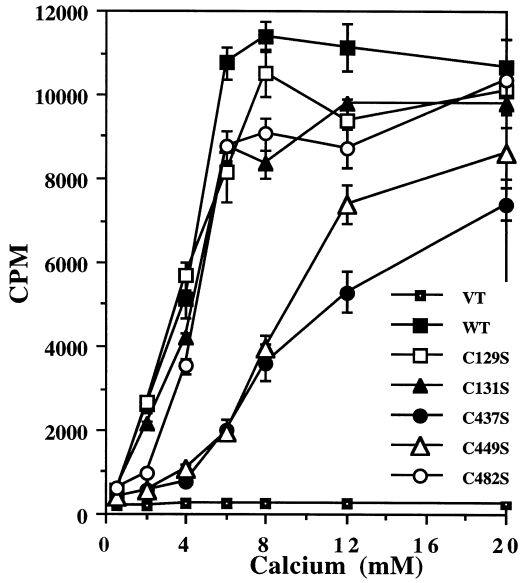

Fig. 3. Concentration dependence for calcium stimulation of phosphoinositide hydrolysis in transiently transfected HEK-293 cells expressing wild-type (WT) and cysteine→serine mutant cDNAs. Transfections were performed, and calcium-stimulated PI hydrolysis assayed as described in Section 2. For each experiment, calcium concentrations were assayed in triplicate. Results are expressed in counts/min of total labeled inositol phosphates generated. Each data point is the mean ± S.E.M. of six independent transfections.

3.4. *Immunoblot analysis of the expression of wild-type and cysteine mutant hCaRs under non-reducing and reducing conditions*

Since the CaR expressed endogenously in rat kidney has been reported to form disulfide-linked dimers [13], we performed immunoblots under reducing and non-reducing conditions of membranes from HEK-293 cells transfected with wild-type and cysteine mutant hCaR cDNAs. As reported previously [14,16], even under reducing conditions (Fig. 4, right) considerable immunoreactivity is detected not only in the size range (130–150 kDa) of monomeric receptor species, but also in a higher size range corresponding to putative dimeric forms of receptor. Under non-reducing conditions (Fig. 4, left), immunostaining of monomers is no longer detectable for wild-type CaR and for cysteine mutants such as C131S which behaves in other assays like wild type or C598S which is non-functional in terms of PI response to calcium. In contrast, C60S and C101S mutants show distinct immunostaining of monomeric CaR and proportionately less immunoreactivity in the dimer size range even under non-reducing conditions.

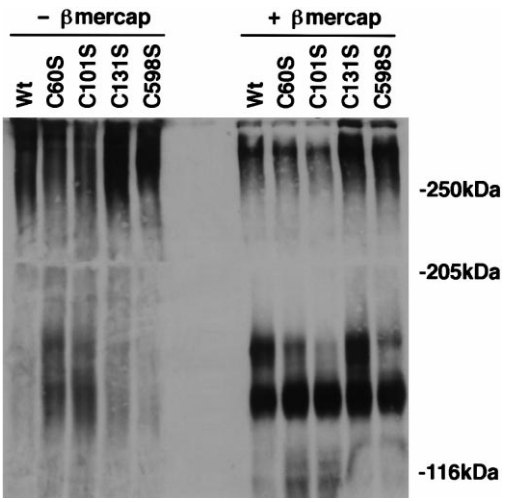

Fig. 4. Immunoblot under non-reducing and reducing conditions showing the expression pattern of wild-type hCaR and cysteine→serine mutants transiently expressed in HEK-293 cells. Crude membrane extracts were prepared and immunoblots performed as in the legend to Fig. 1 except that SDS-PAGE was performed under non-reducing conditions without addition of β-mercaptoethanol (−βmercap) as well as under standard reducing conditions with 2% β-mercaptoethanol (+βmercap).

4. Discussion

There have been numerous previous mutagenesis studies on cysteines in G protein-coupled receptors, but almost all have focused on a pair of disulfide-linked cysteines in the 1st and 2nd extracellular loops. It has been estimated that >90% of G protein-coupled receptors contain cysteines in these two loops, and many studies suggest that disulfide linkage of the 1st and 2nd extracellular loops via these cysteines is important for G protein-coupled receptor structure and function (see for example [18] and references therein).

The CaR and all members of the mGluR subfamily have cysteines in the 1st and 2nd extracellular loops (residues 677 and 765, respectively, in the hCaR). As in other G protein-coupled receptors, these cysteines may be disulfide-linked and may be important for maintaining the structure of the seven membrane-spanning helices, but these cysteines were not the focus of the present study. Instead, we chose to focus on the large number of cysteines located in the amino-terminal ECD, most of which are conserved between the CaR and mGluRs. These conserved cysteines are not found in other G protein-coupled receptors, and no previous studies have examined their functional significance.

Recently, a new family of putative pheromone receptors was cloned from the vomeronasal organ [19–21]. The predicted structure of members of this family is similar to that of the CaR and mGluRs with a large amino-terminal ECD in addition to the seven membrane-spanning helices. Two members of this family, designated mV2R1 and mV2R2, in fact, are more closely related in sequence to the CaR than is any mGluR [19]. In particular, all nine cysteines of the cysteine-rich region are conserved in this new family and four other cysteines (equivalent to residues 236, 358, 395 and 449 in the hCaR) are also conserved in mV2R1 and mV2R2. Given the high degree of conservation of many of the cysteines in the amino-terminal ECD of the CaR, mGluRs and vomeronasal organ putative pheromone receptors, we sought to define the importance of each cysteine in the CaR amino-terminal ECD by mutagenesis and expression studies.

Our results indicate that 14 of these cysteines, including all nine in the cysteine-rich region, are essential for normal receptor expression at the cell surface, and normal function in mediating activation by extracellular Ca^{2+}. Each of these mutant receptors was capable of being expressed as a protein visualized on immunoblots of transfected 293 cell membranes, but in most cases, the protein was primarily in the form of an ~130-kDa band previously shown to reflect an incompletely processed, high mannose form of the receptor (sensitive to endo H sugar cleavage) that does not traffic to the plasma

membrane but is likely retained in the endoplasmic reticulum [14,16]. For some mutants such as cysteines 395 and 546, there appeared to be a decrease even in this form of the protein, perhaps reflecting more rapid proteolytic degradation. The results of cell surface ELISA, a direct measure of receptor expression at the cell surface, in general correlated well with the reduction in an ~150-kDa form of the protein seen on immunoblot. The latter has been shown to represent a fully glycosylated and processed form of the receptor (endo H resistant) that traffics to the cell surface [14,16]. Cell surface ELISA of all mutants was performed with two different ECD monoclonal antibodies with similar results making it likely that reduced ELISA signal reflects decreased cell surface expression rather than failure of antibody to react with mutant forms of the receptor. None of the 14 cysteine mutants was capable of being activated by extracellular Ca^{2+} even at concentrations up to 20 mM. Interestingly, a naturally occurring loss of function mutation of the hCaR, cysteine 582 to tyrosine, was identified in a subject with neonatal severe primary hyperparathyroidism [22]. Functional studies on this mutant were not performed, but our study indicates that cysteine 582 is critical for receptor function.

In contrast, mutation of five other cysteines caused either no significant change in any parameter compared with wild type (C129, C131, C482) or a reduction compared with wild type but with retention of substantial response to Ca^{2+} (C437, C449). Three of the five cysteines that could be mutated without loss of function are the three cysteines in the ECD not conserved between CaR and mGluRs. C131, although conserved between CaR and mGluRs is not conserved in the vomeronasal organ receptors, mV2R21 and mV2R2. Only C449 is conserved in CaR, mGluRs and V2Rs.

Our study defines the critical importance of 14 of the amino-terminal ECD cysteines of the CaR, but does not reveal how substitution of serine for each of these cysteines impairs receptor function. It is tempting to speculate that many of these cysteines are involved in intramolecular disulfide linkages critical for normal cotranslational folding and glycosylation of the amino-terminal ECD as the nascent polypeptide chain is translocated into the lumen of the endoplasmic reticulum. A model for the ECD of the mGluRs (which given the sequence homology can be extended to the CaR) based on the known 3-D structure of bacterial periplasmic binding proteins is compatible with the presence of at least 8–10 cysteines that could be disulfide-linked [23]. The loss of function of the CaR we observe upon mutation of ECD cysteines could be due to loss of normal disulfide linkage with resultant misfolding, aggregation, and retention and degradation in the endoplasmic reticulum.

Failure of mutant receptors to reach the cell surface could by itself account for loss of function, but in some cases loss of function due to abnormal structure could occur even with some mutant receptor reaching the cell surface. Evidence that the CaR [13] and closely related mGluRs form dimers due to disulfide linkage [11,12] suggests that mutation of ECD cysteines could impair intermolecular and not only intramolecular disulfide bond formation. Tryptic proteolysis studies of mGluR5 localized putative intermolecular disulfide(s) involved in dimer formation to the N-terminal 17 kDa of the ECD [11]. Our observation that substantial amounts of C60S and C101S mutants were expressed as monomers even under non-reducing conditions suggests that both of these cysteines within the N-terminal portion of the ECD may be involved in intermolecular disulfides and dimer formation. Given the loss of calcium response of both the C60S and C101S mutants despite significant levels of cell surface expression, particularly for C60S, dimer formation may be critical for signal transduction. Future biochemical studies of the purified receptor and/or ECD will be aimed at defining the number and location of disulfides in the CaR and their specific role in receptor structure and function.

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