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Roles of intraloops-2 and -3 and the proximal C-terminus in signalling pathway selection from the human calcium-sensing receptor

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ABSTRACT

The calcium-sensing receptor (CaSR) couples to signalling pathways via intracellular loops 2 and 3, and the C-terminus. However, the requirements for signalling are largely undefined. We investigated the impacts of selected point mutations in iL-2 (F706A) and iL-3 (L797A and E803A), and a truncation of the C-terminus (R866X) on extracellular Ca^{2+} (Ca_o^{2+})-stimulated phosphatidylinositol-specific phospholipase-C (PI-PLC) and various other signalling responses. CaSR-mediated activation of PI-PLC was markedly attenuated in all four mutants and similar suppressions were observed for Ca_o^{2+}-stimulated $ERK_{1/2}$ phosphorylation. Ca_o^{2+}-stimulated intracellular Ca^{2+} (Ca_i^{2+}) mobilization, however, was relatively preserved for the iL-2 and iL-3 mutants and suppression of adenylyl cyclase was unaffected by either E803A or R866X. The CaSR selects for specific signalling pathways via the proximal C-terminus and key residues in iL-2, iL-3.

1. Introduction

The extracellular Ca^{2+}-sensing receptor (CaSR) belongs to a nutrient-sensing receptor subgroup of G-protein-coupled receptor (GPCR) Class C (reviews: [1,2]). It conforms to a domain-based structure that includes an N-terminus extracellular Venus FlyTrap (VFT) domain linked to a canonical heptahelical signalling domain via an intervening Cysteine-rich domain (reviews: [3,4]). An extended intracellular C-terminal domain of 215 residues provides interactions between the receptor, the cytoskeleton, and some of its key signalling partners (review: [5]). The CaSR acts as a key component of the calciostat that provides feedback regulation of parathyroid hormone secretion (review: [6]). In addition, it is expressed widely in tissues including the kidney, gastro-intestinal tract, bone, brain, and lung in which it plays quite different roles. These include contributions to the control of epithelial transport, hormone secretion, and even cell fate (review: [7]).

The CaSR binds and responds to various endogenous ligands including not only extracellular Ca^{2+} (Ca_o^{2+}) and Mg^{2+} but also organic multivalent cations such as spermine, which acts as an allosteric agonist [8] and L-amino acids, which act as positive modulators (review: [9]) that bind in the receptor's VFT domain [10]. In addition, the CaSR is activated by synthetic modulators (calcimimetics) including the clinically effective phenylalkylamine cinacalcet, which bind in the receptor's heptahelical domain [11].

The finding that the receptor has multiple ligand binding sites and responds to multiple sensing modalities has led us to investigate whether the receptor employs ligand-biased signalling to control function in its diverse cellular contexts. Thus far, these studies have demonstrated that the CaSR exhibits pronounced ligand-biased signalling via pathways coupled to intracellular Ca^{2+} (Ca_i^{2+}) mobilization, $ERK_{1/2}$ phosphorylation and membrane ruffling [12], and that mutations associated with disturbed CaSR function in vivo perturb the normal balance between different signalling pathways with respect to the potencies and even efficacies of Ca_o^{2+} and other activators [13,14]. The findings indicate that signalling bias is an important property of the receptor that can explain

Abbreviations: GPCR, G-protein coupled receptor; CaSR, calcium-sensing receptor; PI-PLC, phosphatidylinositol-specific phospholipase-C; VFT, Venus FlyTrap; CR, Cysteine-rich; iL, intraloop; cAMP, adenosine 3′,5′-cyclic monophosphate; pERK, phosphorylated extracellular regulated kinase; IP_1, inositol 1-phosphate

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key differences in receptor function between tissues and also in the patterns of disease arising from specific disease-related mutations.

Signalling bias arises from the adoption of specific ligand-bound receptor conformations that select between signalling pathways, pointing to the existence of distinct molecular requirements for the activation of different G-proteins and their downstream signalling pathways. In response to stimulation by its principal physiological agonist, Ca_o^{2+}, for example, the CaSR couples to various intracellular signalling responses including $G_{q/11}$-mediated activation of PI-PLC, Ca_i^{2+} mobilization, $G_{i/o}$-mediated inhibition of adenylyl cyclase, and thus suppression of intracellular cAMP levels (review: [6]), as well as the phosphorylation of various protein kinases including the MAP kinase $ERK_{1/2}$ ($pERK_{1/2}$) [15,16].

In the present study, we set out to investigate whether it is possible to define distinct subsets of molecular requirements for the Ca_o^{2+}-stimulated activation of $G_{q/11}$, $G_{i/o}$ and $ERK_{1/2}$ phosphorylation, taking advantage of previous work on the molecular requirements of Ca_o^{2+}-stimulated PI-PLC activation by the bovine CaSR [17], which exhibits 93% amino acid identity with the human CaSR (hCaSR). This work identified the conserved residue F707 (hCaSR residue F706) in iL-2, and three mutants homologous to hCaSR L797A, F801A and E803A, all of which exhibited marked attenuations in their Ca_o^{2+}-stimulated PI-PLC responses [17]. Furthermore, truncation of the proximal C-terminus beyond hCaSR residue S865, by the introduction of a premature stop codon R866X, abolished PI-PLC signalling [18,19].

Thus, we investigated the impacts of four human CaSR mutants on Ca_o^{2+}-stimulated signalling responses including: PI-PLC as reported by IP_1 accumulation; Ca_i^{2+} mobilization; $pERK_{1/2}$; and suppression of adenylyl cyclase. The results indicate that there are distinct G-protein coupling requirements for CaSR-mediated $G_{q/11}$ and $G_{i/o}$ activation and that these differences are important for signalling pathway selection in response to elevated Ca_o^{2+}. Since distinct ligands exhibit significant differences in CaSR-mediated signalling pathway selection the current findings would also appear to provide insights into the nature of biased signalling responses.

2. Materials and methods

2.1. Construction of mutant receptors

The wild-type (WT) human CaSR cDNA (cassette version, [20]) cloned between the *Kpn* I and *Xba* I sites of pcDNA3.1 (+) {pcDNA3.1 (+)-WTCaSR} was a kind gift from Dr. Mei Bai and Prof. Edward Brown (Endocrine-Hypertension Division and Membrane Biology Program, Brigham and Women's Hospital, Boston, MA, USA). All mutants were generated in pcDNA3.1(+)-WTCaSR and/or pcDNA3.1(+)-WTCaSR(FLAG) plasmid, which contains the FLAG epitope DYKDDDDK between residues 371 and 372; insertion of the FLAG epitope at this position has been shown previously to have no impact on receptor function [21]. The Quikchange II site-directed mutagenesis protocol was used to introduce the point mutations F706A, L797A and E803A. Briefly, pairs of complementary or overlapping primers (30–40 bases) were designed to encode the required mutation with flanking wild-type sequences of around 15–20 bases. The template DNA was amplified for 18 cycles with Pfu Ultra II HS DNA polymerase (Agilent Technologies, USA). Following digestion with *Dpn* I, amplified DNA was transformed into DH5α *Escherichia coli* cells. Sequences of forward and reverse primers used to generate the required point mutations are shown in Table 1.

A truncation mutant, R866X, which introduces a premature stop codon after S865 in the proximal C-terminus, was also generated. Briefly, the template cDNA was amplified by PCR using a forward primer designed to bind to the first 22 nucleotides of the CaSR cDNA with a 5′ *Kpn* I site (5′-CAG TAT GGT ACC ATG GCA TTT TAT AGC TGC TGC T) and a reverse primer (5′-TAGACT TCT AGA TTA GGA TGG CTT GAA GAG AAT GAT) that introduced a stop codon (TAA) at residue 866 (underlined) followed by a 3′ *Xba* I site. The PCR product obtained using the wild-type CaSR as the template was digested with *Kpn* I and *Xba* I and ligated into the multiple cloning site of purified pcDNA3.1(+) that had been pre-digested using these enzymes. The identities of all completed mutants were confirmed by DNA sequencing (Australian Genome Research Facility, Sydney, NSW, Australia).

Table 1
Sequences of forward and reverse primers used to generate the CaSR point mutations tested in the study. The mutated codon is underlined.

Mutant	Primers used in site-directed mutagenesis reactions
F706A	F: 5′-CCAACCGTGTCCTCCTGGTGGCTGAGGCCAAGAT-3′ R: 5′-TGGGGATCTTGGCCTCAGCCACCAGGAGGACA-3′
L797A	F: 5′-TTCAAGTCCCGGAAGGGCGCCGGAGAACTTCAATGAA-3′ R: 5′-AAGTTCTCCGGCGCCTTCCGGGACTTGAAGGCAAA-3′
E803A	F: 5′-GCCGGAGAACTTCAATGCAGCCAAGTTCATCACCTTCAG-3′ R: 5′-GTGATGAACTTGGCTGCATTGAAGTTCTCCGGCAGCTTC-3′

2.2. Cell culture and transfection

HEK-293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 25 Units/ml Penicillin and 25 μg/ml of Streptomycin, and maintained at 37 °C in a humidified 5% CO_2 incubator. When cells had reached 85–95% confluency they were transfected with XtremeGENE HP transfection reagent (Roche, Germany). Briefly, 0.5–1 μg samples of WT or mutant DNA in 1–2 μl of water and 3 μl of transfection reagent were diluted with 100 μl of DMEM and allowed to complex at RT for 15 min. The transfection solution was added to the cell cultures to a final concentration of 9.1% (v/v). In all experimental series, identical DNA concentrations were used for all constructs tested (i.e., WT and all four mutants). For cAMP measurements, 0.5 μg samples of cAMP reporter construct DNA were added along with the WT or mutant DNA to the transfection solutions.

2.3. Quantitation of total and surface receptor expression

HEK-293 cells were cultured in 96-well poly-D-lysine coated plates and transiently transfected for 48 h with either the wild-type CaSR or one of several mutant CaSRs. After transfection, cell samples, at an approximate density of 100% (4×10^4 cells $well^{-1}$), were washed once with TBS-T (0.05 M Tris, 0.15 M NaCl, 0.05% (v/v) Tween-20, pH 7.4) and fixed for 15 min on ice with either 4% (w/v) paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) to determine surface expression, or methanol, to determine total cell expression. All subsequent steps were performed at room temperature. After washing once with TBS-T, fixed cell samples were incubated with 1% (w/v) skim milk solution in TBS-T for 1 h and then incubated with monoclonal anti-FLAG M2 horse-radish peroxidase (HRP)-conjugated antibody (Sigma Aldrich #A8592) diluted 1:5000 in TBS-T for 1 h. The wells were then washed three times with PBS and incubated with the HRP substrate, 3,3′,5,5′ tetramethylbenzidine liquid substrate solution (Sigma Aldrich cat. #T0440) in the dark for 12 min. Enzyme reactions were stopped by the addition of equal volumes of 1 M HCl. Supernatant samples were transferred to new 96-well plates and A_{450} values were obtained using a Perkin-Elmer EnVision 2103 multilabel counter (software version 1.08, Perkin Elmer, USA).