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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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\(^{2+}\))-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\(^{2+}\)-stimulated ERK1,2 phosphorylation. Ca\(^{2+}\)-stimulated intracellular Ca\(^{2+}\) (Ca\(^{2+}\)) 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.

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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 calcistat 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.

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\(_i\), inositol 1-phosphate

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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\(^{2+}\)) 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 heptahelial 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\(^{2+}\)) 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\(^{2+}\) and other activators [13,14]. The findings indicate that signalling bias is an important property of the receptor that can explain...
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 physiological agonist, CaSR, for example, the CaSR couples to various intracellular signalling responses including Gq/11-mediated activation of PI-PLC, Ca2+ mobilization, Gi/o-mediated inhibition of adenyl cyclase, and thus suppression of intracellular cAMP levels (review: [6]), as well as the phosphorylation of various protein kinases including the MAP kinase ERK1/2 ([15,16]).

In the present study, we set out to investigate whether it is possible to define distinct subsets of molecular requirements for the CaSR-stimulated activation of Gq/11, Gi/o and ERK1/2 phosphorylation, taking advantage of previous work on the molecular requirements of CaSR to Gq/11-mediated 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 CaSR-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 CaSR-stimulated signalling responses including: PI-PLC as reported by IP; accumulation; Ca2+ mobilization; ERK1/2; and suppression of adenyl cyclase. The results indicate that there are distinct G-protein coupling requirements for CaSR-mediated Gi/o activation and that these differences are important for signalling pathway selection in response to elevated Ca2+. 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 Quickchange 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 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-3’ and a reverse primer (5’-TAGACT TCT AGA TTA GGA TGG CTT GTA AAG AAT GAT-3’) 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).

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% CO2 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 transfaction 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 solution.

2.3. Quantification of total and surface receptor expression

HEK-293 cells were cultured in 96-well polystyrene 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 × 104 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 Na2HPO4, 1.8 mM KH2PO4, 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. Supematant samples were transferred to new 96-well plates and A450 values were obtained using a Perkin-Elmer EnVision 2103 multilabel counter (software version 1.08, Perkin Elmer, USA).

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primers used in site-directed mutagenesis reactions</th>
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<tbody>
<tr>
<td>F706A</td>
<td>F: 5’-CCACCGTGGTCTCTTCTGGTGCCTGACGCGCAATGAT-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-TGGCGATTGGTGGTCCGCCACCGACGAGCA-3’</td>
</tr>
<tr>
<td>L797A</td>
<td>F: 5’-TCCAGTCCCACCGAGGCCACCCGGAGAACCTATGCAA-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-AAGTCTCTGGCCCTGGCCGATTGACGAGCC-3’</td>
</tr>
<tr>
<td>E803A</td>
<td>F: 5’-GCCGGAGAATCCTATCCGACCAATCCCTACCTGCAG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GTATTCACTGGGCTGATTAAGTGGCTTACCTGCAG-3’</td>
</tr>
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Table 1. Sequences of forward and reverse primers used to generate the CaSR point mutations tested in the study. The mutated codon is underlined.
2.4. Homogeneous time-resolved fluorescence assay for inositol monophosphate (IP$_1$)

HEK-293 cells were cultured in 6-well plates and transfected at 90% confluency for 24 h. Cell samples (from ca. 1.2 × 10$^6$ cells) were detached with 0.2 ml lots of 0.25% trypsin-EDTA, followed by the addition of 5–10 ml DMEM (10% FBS; 25 Units/ml Penicillin, 25 µg/ml Streptomycin) and centrifugation (210 × g, 3 min). Cell pellets were re-suspended in 1.2 ml of DMEM (10% FBS, 25 Units/ml Penicillin, 25 µg/ml Streptomycin), and 30 µl samples of cell suspensions were aliquoted into 384 well Optitplates (Perkin Elmer, USA) and cultured for 24 h. On the day of the experiment, the medium was removed from each well and the cells were incubated for 30 min in 14 µl lots of stimulation buffer that contained 146 mM NaCl, 4.2 mM KCl, 5.5 mM glucose, 0.5 mM MgCl$_2$, 10 mM HEPES (pH 7.4), and 50 mM LiCl to suppress IP$_1$ breakdown. IP$_3$ was detected by overnight incubation with 3 µl/well of anti-IP$_3$ cryptate Tb conjugated antibody (diluted in 1 × lysis buffer according to the manufacturer’s instructions) and 3 µl/well of similarly prepared IP$_3$-d2 conjugate. Time-resolved PERT was measured on a Perkin-Elmer EnVision 2103 multilabel counter with excitation at 320 nm and emission at 615 and 665 nm. The F$_{340}$/F$_{665}$ emission ratio was used as a measure of IP$_3$.

2.5. Cytoplasmic Ca$^{2+}$ mobilization fluorescence assay

Microfluorimetry to measure changes in Ca$^{2+}$ was performed as described previously [22]. All Ca$^{2+}$ solutions were prepared in physiological saline solution (PSS) that contained 125 mM NaCl, 4 mM KCl, 0.1% (w/v) D-Glucose, 1 mM MgCl$_2$, 20 mM HEPES (pH 7.45 adjusted with NaOH). Briefly, HEK-293 cells that had been cultured on 15 mm sterile glass coverslips in 24-well plates were transfected with either the WT CaSR or one of several CaSR mutants for 48 h. After transfection, the cells were rinsed with PSS supplemented with 0.8 mM NaH$_2$PO$_4$ and 0.1% (w/v) bovine serum albumin (BSA) and then incubated with this solution containing 5 µM Fura 2-AM for 7 min each. In control experiments, 7 min was found to be an appropriate time for the adoption of a new steady-state level of cAMP. The emission ratios arising from the last one-minute intervals were averaged and after background subtraction, the behaviour of the WT CaSR at baseline Ca$^{2+}$ was determined using the AlphaScreen protocol in a Perkin-Elmer EnVision 2103 multilabel counter. To generate Ca$^{2+}$ concentration–response curves, raw data were expressed as fold-changes with respect to the behaviour of the WT CaSR at baseline Ca$^{2+}$ (0.5 mM).

2.7. Intracellular cAMP assay

Microfluorimetry was performed to detect changes in intracellular cAMP levels as described previously [22]. Briefly, HEK-293 cells were seeded onto sterile glass coverslips in 24-well plates. The cells were then transfected with either the WT CaSR or one of several mutant CaSR constructs, and co-transfected with the cAMP fluorescence resonance energy transfer reporter construct: CFP-EPAC-YFP-YFP [22]. After 48 h, the medium was removed from each well and the cells were incubated with 0.5 mM Ca$^{2+}$ in PSS for 15 min at 37 °C. The coverslips were then transferred individually to a closed bathing chamber and placed in the light path of the Zeiss Axiovert 200 M microscope described above. The cells were then exposed to PSS that contained 1 µM Forskolin for 15 min to elevate intracellular cAMP levels in the presence of baseline Ca$^{2+}$ (0.5 mM) followed by stepwise increments in Ca$^{2+}$ (0.5–10 mM) for 7 min each. In control experiments, 7 min was found to be an appropriate time for the adoption of a new steady-state level of cAMP. Excitation was performed at a constant wavelength of 436 nm and emission was performed at alternating wavelengths of 488 and 528 nm for intervals of 0.5 s corresponding to F$_{488}$ and F$_{528}$, respectively. Images were collected using the AxioCam HSM digital camera described above in Section 2.5 and computed by Slidebook software as the F$_{488}$/F$_{528}$ emission ratio as a measure of intracellular cAMP levels. The emission ratios arising from the last one-minute intervals of exposure were averaged and after background subtraction (0.5 mM Ca$^{2+}$ control) were expressed as percentages of the Forskolin-stimulated emission ratio to generate Ca$^{2+}$ concentration–response curves.

2.8. Curve fitting and statistical analysis

The data are expressed routinely as means ± S.E.M. For Ca$^{2+}$ mobilization experiments, Ca$^{2+}$ concentration–response data were obtained by integrating the F$_{340}$/F$_{380}$ excitation ratio data obtained in response to each Ca$^{2+}$ concentration as described previously [22]. Concentration–response data for IP$_3$ accumulation, Ca$^{2+}$ mobilization and pERK$_{1/2}$ were fitted to the following form of the Hill equation:

\[
R = d + a \cdot (d - b) \cdot C^b \div (e^b + C^b)
\]

where \(a\) = maximal response (\(E_{\text{max}}\)); \(b\) = Hill coefficient; \(e\) = half maximal effective concentration (EC$_{50}$); \(d\) = minimum response. Concentration–response data for suppression of intracellular cAMP levels were fitted using the following equation:

\[
R = a - (a - d) \cdot C^b \div (e^b + C^b)
\]
where the parameters $a$, $b$, $d$, and $e$ have the same meanings as in Eq. (1).

Statistical analyses were performed using analysis of variance (ANOVA) in GraphPad Prism 5. Statistical significance was accepted at $P < 0.05$. Log transformations of $EC_{50}$ or $IC_{50}$ values were used to assess the statistical significance of differences in $Ca^{2+}$-sensitivity.

3. Results

3.1. Construction and expression of PLC signalling defective CaSR mutants

To assess the impacts of the PI-PLC-signalling defective mutants on various CaSR-mediated signalling pathways, we generated three alanine mutants in CaSR iL-2 (F706A) and iL-3 (L797A and E803A) as well as a C-terminus truncation mutant, R866X, which retains only the three membrane-proximal residues (863-KPS-865) of the normal 216 residue C-terminus (Fig. 1A). The constructs were transiently transfected into HEK-293 cells to assess expression and function. Levels of total and cell surface expression for all four mutants were comparable to WT as determined by anti-FLAG antibody-based ELISAs (Fig. 1B).

3.2. The impacts of iL-2, iL-3 and C-terminus truncation mutants on CaSR-mediated IP$_1$ accumulation

The PI-PLC signalling properties of the mutants were assessed by IP$_1$ accumulation in transiently-transfected HEK-293 cells exposed to stepwise increases in $Ca^{2+}$ in the range 0.5–20 mM (Fig. 2A). A homogeneous time-resolved FRET (HTRF)-based competitive assay was used to assess IP$_1$ levels over 30 min. WTCaSR-transfected HEK-293 cells responded to elevated $Ca^{2+}$ with increased IP$_1$ accumulation and a maximally effective $Ca^{2+}$ of 20 mM; a 4-fold increase in IP$_1$ was observed with respect to the baseline response at 0.5 mM $Ca^{2+}$.

All mutant CaSR-expressing cells exhibited markedly impaired IP$_1$ responses that were significantly reduced at 20 mM $Ca^{2+}$ compared to WT ($P < 0.0001$; 2-way ANOVA). The maximal responses of HEK-293 cells expressing E803A and R866X were around 30%, and the maximal responses of HEK-293 cells expressing F706A and

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**Fig. 1.** PI-PLC signalling-defective mutants in iL-2, iL-3 and the C-terminal region, and their impact on receptor expression. (A) The residues that were mutated are shown in black in both iL-2 (F706) and iL-3 (L797 and E803). A mutation (R866X) that truncates the C-terminal region after S865 (red) was also generated. All four mutants have been reported previously to impair PI-PLC signalling [17,19]. (B) Effects of iL point mutations and a C-terminus truncation mutant on CaSR total and surface expression. HEK-293 cells were transiently transfected with FLAG-tagged WT or mutant constructs, and then fixed and probed with anti-FLAG antibody to measure total (h+j) or cell surface (j) expression as described in the Section 2. Results obtained in HEK-293 cells transfected with vector alone were used for background subtraction and the results for all mutants were normalised to the level of total WTCaSR expression. The results were obtained in four independent experiments.

**Fig. 2.** Impact of CaSR mutants on $Ca^{2+}$-stimulated IP$_1$ accumulation and $Ca^{2+}$ mobilization. HEK-293 cells were transiently transfected with the WTCaSR (●), or one of several CaSR mutants including R866X (○), F706A (□), L797A (▲), or E803A (▼). In (A), they were exposed to various $Ca^{2+}$ concentrations prior to lysis, and then processed for IP$_1$ accumulation as described in the Section 2 (4–5 independent experiments). In (B), transfected cells that had been grown on coverslips were loaded with fura-2 AM and mounted in a perfusion chamber in the light path of a Zeiss Axiovert microscope for live cell imaging, after which they were exposed to step-wise increments in $Ca^{2+}$ from 0.5 to 20 mM. F$_{540}$/F$_{380}$ excitation ratio data were integrated to allow the generation of concentration–response curves (9–10 cells in each of 4–9 independent experiments).
3.3. The impacts of iL-2, iL-3 and C-terminus truncation mutants on CaSR-mediated Ca\textsuperscript{2+} mobilization

We next investigated the impacts of the mutants on Ca\textsuperscript{2+} mobilization in single Fura-2-loaded HEK-293 cells by microfluorimetry. The WT CaSR induced Ca\textsuperscript{2+}-dependent Ca\textsuperscript{2+} mobilization with an EC\textsubscript{50} for Ca\textsuperscript{2+} of 2.9 ± 0.2 mM and an E\textsubscript{max} of 13.8 ± 0.4 IFRU, and the C-terminus truncation mutant R866X abolished Ca\textsuperscript{2+}-dependent Ca\textsuperscript{2+} mobilization (Fig. 2B). In the cases of all three iL point mutants studied, however, Ca\textsuperscript{2+} mobilization was relatively preserved. Thus, there were only approximately 50% reductions in E\textsubscript{max} values (all P < 0.01) and the EC\textsubscript{50} values for Ca\textsuperscript{2+} were similar to and not significantly different from WT (P > 0.1 in all cases; Fig. 2B). In particular, the EC\textsubscript{50} values for Ca\textsuperscript{2+} in the case of the iL-2 mutant F706A was 5.3 ± 0.6 mM and in the cases of the iL-3 mutants L797A and E803A were 6.8 ± 1.4 and 3.0 ± 0.6 mM respectively. Thus, the impacts of the iL-2 and iL-3 mutants were less marked on Ca\textsuperscript{2+} mobilization than on IP\textsubscript{3} accumulation, although Ca\textsuperscript{2+} mobilization was abolished in HEK-293 cells that were transiently transfected with R866X.

3.4. The impacts of iL-2, iL-3 and C-terminus truncation mutants on CaSR-mediated phosphorylation of ERK1/2

The impacts of the mutants on Ca\textsuperscript{2+}-stimulated ERK1/2 phosphorylation (pERK1/2) were investigated using a dual antibody AlphaScreen assay as described in the Section 2. In HEK-293 cells expressing the WT CaSR, elevated Ca\textsuperscript{2+} induced concentration-dependent increases in pERK1/2 to a maximum 10–12-fold above the baseline level observed at a Ca\textsuperscript{2+} of 0.5 mM. The EC\textsubscript{50} for Ca\textsuperscript{2+} was 2.4 ± 0.3 mM (Fig. 3). The C-terminus truncation mutant did not support Ca\textsuperscript{2+}-stimulated pERK1/2 at concentrations up to 10 mM. In addition, the three point mutations F706A, L797A and E803A markedly suppressed the E\textsubscript{max} values by ≥90% with respect to the WT CaSR (P < 0.001 in all three cases). The results indicate that Ca\textsuperscript{2+}-stimulated CaSR-mediated pERK1/2 is critically dependent on residues in iL-2 and iL-3 that are required for PI-PLC activation along with residues that lie to the C-terminus side of S865.

3.5. The impacts of iL-2, iL-3 and C-terminus truncation mutants on CaSR-mediated suppression of adenylyl cyclase as reported by cAMP levels

We next examined the impacts of the iL-2, iL-3 and C-terminus truncation mutants on CaSR-mediated suppression of adenylyl cyclase as reported by cAMP levels measured in individual cells by microfluorimetry. Initial experiments demonstrated that Ca\textsuperscript{2+}-dependent suppression of intracellular cAMP levels but not Ca\textsuperscript{2+}-dependent Ca\textsuperscript{2+} mobilization was abolished after overnight pre-exposure to pertussis toxin (300 ng mL\textsuperscript{-1}) and was insensitive to the phosphodiesterase inhibitor IBMX (0.2 mM), thereby demonstrating that Ca\textsuperscript{2+}-mediated inhibition of adenylyl cyclase was the major determinant of Ca\textsuperscript{2+}-stimulated suppression of cAMP levels in these experiments (not shown). HEK-293 cells were transiently co-transfected with a cAMP reporter construct and one of the four CaSR mutants, and studied by microfluorimetry as described in the Methods section. Samples of transfected cells were exposed to 1 μM forskolin to elevate intracellular cAMP levels followed by step-wise increments in Ca\textsuperscript{2+} from 0.5 to 10 mM. HEK-293 cells transfected with the WT CaSR exhibited Ca\textsuperscript{2+}-dependent suppression of 1 μM forskolin-stimulated cAMP levels with an EC\textsubscript{50} for Ca\textsuperscript{2+} of 1.7 ± 0.2 mM and a maximal 60% suppression of cAMP levels (Fig. 4). Two of the point mutations, F706A and L797A abolished Ca\textsuperscript{2+}-dependent cAMP suppression (Fig. 4). However, surprisingly, the Ca\textsuperscript{2+}-stimulated inhibitory responses were maintained in HEK-293 cells transfected with either the iL-3 point mutation E803A or the C-terminus truncation mutant R866X. In the case of R866X, there was no apparent change in the EC\textsubscript{50} for Ca\textsuperscript{2+}. In the case of E803A, an apparent drop in EC\textsubscript{50} for Ca\textsuperscript{2+} from 1.7 ± 0.2 to 0.9 ± 0.1 mM (Fig. 4) was not statistically significant (P = 0.07). The findings demonstrate that the molecular requirements for Ca\textsuperscript{2+}-stimulated CaSR-mediated inhibition of cAMP levels are clearly distinct from those required for the control of PI-PLC, Ca\textsuperscript{2+} mobilization and pERK1/2 and provide new insights into the nature of biased signalling.

4. Discussion

Within GPCR class C there is a sub-group of nutrient-sensing receptors that respond to both macronutrients, such as t-amino acids, as well as micronutrients, such as Ca\textsuperscript{2+} ions (review: [2]).
The CaSR is a key member of this receptor subgroup that plays diverse physiological roles. These include the feedback control of calcium homeostasis via cell-based mechanisms in the parathyroid, renal thick ascending limb, and bone [24], the modulation of tissue development and cell fate [7], and L-amino acid sensing mechanisms [25] and various other functions in the gastro-intestinal tract (review: [26]). This surprising pluriptency requires precise control over a large number of receptor-coupled signalling pathways, and, thus, mechanisms by which the receptor can select between pathways.

In the present study, we focused on Ca\textsuperscript{2+}-stimulated control of four key CaSR-mediated signalling pathways: PI-PLC, Ca\textsuperscript{2+} mobilization; ERK\textsubscript{i,j} phosphorylation; and suppression of adenyl cyclase, using key point mutants of CaSR iL-2 and iL-3, as well as a truncation mutant of the proximal C-terminus to assess the roles of these structural components in the selection of distinct signalling pathways. We used transient expression of the five different CaSR constructs in the present study to ensure that the cell context was identical in all cases. None of the mutants impaired either total or surface expression of the receptor, and all of them exhibited marked suppression of Ca\textsuperscript{2+}-stimulated PI-PLC (Figs. 1B and 2A) as reported previously [17,19]. Strikingly, we found that the C-terminal-proximal region is required for the activation of PI-PLC, Ca\textsuperscript{2+} mobilization and pERK\textsubscript{i,j} but not for G\textsubscript{i/o}-dependent inhibition of adenyl cyclase, as reported by the suppression of forskolin-stimulated elevations in cAMP levels. In addition, we observed important differences in the roles of key iL-2 and iL-3 residues in supporting signalling by distinct pathways. Thus, although F706 and L797 were required for both G\textsubscript{i/o} and G\textsubscript{i}-dependent signalling, E803 was only required for G\textsubscript{i/o}, and not G\textsubscript{i}-dependent signalling. The results indicate that distinct residues in these key intracellular regions of the CaSR are required by the mechanisms that engage specific pathways in response to Ca\textsuperscript{2+}-stimulation. See Table 2 for a summary of the outcomes.

All mutants markedly impaired Ca\textsuperscript{2+}-stimulated pERK\textsubscript{i,j} as well as PI-PLC suggesting the existence of a common signalling mechanism downstream of G\textsubscript{i/o}. Ca\textsuperscript{2+}-stimulated CaSR-mediated pERK\textsubscript{i,j} was nearly abolished by the PI-PLC inhibitor, U73122, and also markedly suppressed following pre-treatment with pertussis toxin, which disables G\textsubscript{i/o} [15,27]. Thus, both G\textsubscript{i/o}-stimulated PI-PLC and G\textsubscript{i/o} appear to be required for pERK\textsubscript{i,j}.

Surprisingly, there were notably lesser impacts of the three point mutants on Ca\textsuperscript{2+} mobilization when compared with their effects on IP\textsubscript{3} accumulation (Fig. 2B) and only the C-terminus truncation mutant R866X abolished Ca\textsuperscript{2+} mobilization. Thus, the results suggest only a partial dependence of CaSR-mediated Ca\textsuperscript{2+} mobilization on PI-PLC. Other pathways that may contribute to CaSR-mediated Ca\textsuperscript{2+} mobilization include a G\textsubscript{12/13}-coupled pathway dependent on a Rho and Filamin-A-dependent interaction with the receptor’s C-terminus [28]. Such a mechanism could explain how the C-terminal truncation mutant R866X abolished Ca\textsuperscript{2+} mobilization. G\textsubscript{12} has also been reported to activate protein phosphatase 2A [29], an enzyme that supports Ca\textsuperscript{2+}-stimulated dephosphorylation of CaSR residue T888 and, in turn, promotes stable elevations in Ca\textsuperscript{2+} [30].

Preliminary experiments investigating the effects of pretreatment with pertussis toxin and of acute exposure to the broad-spectrum phosphodiesterase (PDE) inhibitor IBMX demonstrated that the suppressive effect of Ca\textsuperscript{2+} on forskolin-induced elevations in cAMP levels was dependent on G\textsubscript{i/o}-mediated inhibition of adenylyl cyclase and not PDE. Thus, the finding that F706A (iL-2) and L797A (iL-3) were resistant to Ca\textsuperscript{2+}-stimulated suppression of cAMP is consistent with the idea that both these residues are required for CaSR-mediated coupling to G\textsubscript{i/o} as well as G\textsubscript{i}. However Ca\textsuperscript{2+}-stimulated suppression of cAMP levels was retained at apparently WT levels in the cases of the point mutant E803A (iL-3) and the C-terminus truncation mutant R866X, demonstrating the existence of distinct requirements for the activation of PI-PLC and inhibition of adenylyl cyclase. In addition, the finding that R866X abolished Ca\textsuperscript{2+}-stimulated Ca\textsuperscript{2+} mobilization but exhibited WT levels of Ca\textsuperscript{2+}-stimulated suppression of cAMP appears to exclude a role for Ca\textsuperscript{2+}-dependent inhibition of adenylyl cyclase in the present experiments in which forskolin was used to elevate cAMP levels, distinct from that reported previously for CaSR-expressing HEK-293 cells in which they were elevated by prostaglandin E\textsubscript{2} or isoproterenol [31].

The finding that CaSR-mediated coupling to the control of PI-PLC and adenylyl cyclase is dependent on distinct residues is consistent with findings on the signalling requirements of another class C GPCR, mGlur1, in which some iL-2 mutants, including T695A, K697A and S702A, selectively disabled PI-PLC activation, whereas others including P698A and the two residue deletion C694-T695, selectively impaired cAMP accumulation [32]. Similarly, deletion of the mGlur1 C-terminus selectively abolished G\textsubscript{i/o}, but not G\textsubscript{i}-dependent signalling [33] as observed in the present study for the Ca\textsuperscript{2+}-stimulated CaSR (Fig. 4).

The C-terminus also plays important roles in coupling other class C GPCRs to specific G-protein-dependent signalling pathways [34]. For example, alternative splicing of mGlur1 results in distinct G-protein coupling profiles based on differences in C-terminus sequences. Thus, mGlurR-1\textsubscript{a} with a longer C-terminus coupled efficiently to both G\textsubscript{i/o} and G\textsubscript{i}, whereas mGlurR-1\textsubscript{b} with a shorter C-terminus coupled efficiently to G\textsubscript{i/o} but did not couple to G\textsubscript{i} [35]. Furthermore, deletion of the mGlur1 C-terminus selectively abolished G\textsubscript{i/o}-dependent signalling but not G\textsubscript{i}-dependent signalling [33].

It is notable in the present study that where one or more of the iL-2, iL-3 or C-terminus mutants impaired CaSR-mediated stimulation of PI-PLC, Ca\textsuperscript{2+} mobilization, ERK\textsubscript{i,j} phosphorylation, and/or inhibition of adenylyl cyclase, the primary effect was a reduction in the maximal response (E\textsubscript{max}) rather than an increase in the EC\textsubscript{50} value for Ca\textsuperscript{2+} (see Figs. 2–4), as more commonly reported for mutations in human kindreds with familial hypocalciuric hypercalcemia (FHH) or neonatal severe primary hyperparathyroidism (NSHPT) (reviews: [36–38]). The finding that E\textsubscript{max} was reduced is consistent with the idea that Ca\textsuperscript{2+} binding and Ca\textsuperscript{2+}-dependent changes in receptor conformation were normal in the extracellular VFT and Cyt-rich domains, but that coupling of the activated receptor to its signalling apparatus in the dimeric heptahelical domains was defective as a result of impaired binding of partner proteins including G-proteins and enzymes.

In the present study we have identified CaSR residues in iL-2 and iL-3 as well as the proximal C-terminus that are required for Ca\textsuperscript{2+}-stimulated signalling, and demonstrated the existence of distinct requirements for the activation of PI-PLC and Ca\textsuperscript{2+} mobilization, and suppression of adenylyl cyclase. Further work is required to properly define the requirements of Ca\textsuperscript{2+}-stimulated pathways downstream of G\textsubscript{i/o}, G\textsubscript{i}, and G\textsubscript{12/13}, and to assess the ligand-biased signalling requirements of physiologically and pharmacologically...
important agonists including divalent cations such as Mg$^{2+}$ and Sr$^{2+}$ and organic polycations such as spermine, as well as modulators including $L$-amino acids and cinacalcet.

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References