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G-protein Coupling & Subunit
Requirements in Ca$^{2+}$-sensing Receptor
Signalling

Mahvash Ayesha Goolam

Supervisor: Prof. Arthur Conigrave

A thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

School of Molecular Bioscience
University of Sydney
Australia

2013
Declaration

I hereby declare that the data presented in this thesis are from original work that I have carried out from February, 2008, to March, 2013, for my Ph.D project. No part of the data has been presented for the purpose of an award of any other degree in any university.

Part of the data has been published as conference abstracts.

Mahvash Ayesha Goolam

(signed)
Acknowledgements

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Publications

Journal Articles


Scientific Presentations

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Brennan SC, Khan MA, Windloch KA, Christopoulos A & Conigrave AD. “Analysis of the roles of N-linked glycosylation sites in the Calcium–sensing Receptor.” ComBio Meeting held in Christchurch, New Zealand in December, 2009

Khan MA, Whan R, Kable EW, Mun H-C, Braet F, and Conigrave AD. “FRET between CaR Dimers labelled in intracellular loop-2.” Hunter Valley Cell Biology Meeting held in the Hunter Valley, Australia in March, 2010

Khan MA, Chan R, Conigrave AD. “Expression of Ca$^{2+}$-sensing Receptor Heterodimers at the Cell Surface using the GABA$\beta$ sorting system.” OzBio meeting held in Melbourne, Australia in September, 2010

Khan MA, Chan R, Christopoulos A, Conigrave AD. “Ca$^{2+}$ sensitivity of Mutant Ca$^{2+}$-sensing Receptor Heterodimers.” Molecular Pharmacology of GPCR meeting held in Melbourne, Australia in December, 2010.

Khan MA, Chan R, Christopoulos A, Conigrave AD. “Minimal binding site requirements for activators and modulators of the Ca$^{2+}$ sensing receptor.” ComBio meeting held in Cairns, Australia in September, 2011. Awarded student poster prize by Australian Society for Biochemistry and Molecular Biology.

Goolam MA, Ward JH and Conigrave AD. “The role of calcium-sensing receptor intracellular loops and C-tail in G$_{q/11}$ and G$_{i/o}$ signalling.” Austrian Society for Bone and Mineral Research Annual meeting held in Vienna, Austria in November, 2012.
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Abstract

The human Ca\(^{2+}\)\(_o\)-sensing receptor (CaSR) is a class C GPCR that responds to physiological ligands, including Ca\(^{2+}\)\(_o\) and L-amino acids, by modulating a variety of signalling pathways that induce changes in intracellular enzymes and metabolites including phospholipases, MAP kinases, Ca\(^{2+}\), and cAMP. Very little is known about the upstream events, such as G-protein coupling and intersubunit interactions that result in the CaSR regulating signalling pathways in response to ligand binding. The work presented in this thesis provides insight into the role of intracellular residues and domains as molecular determinants of G-protein coupling and specificity. Furthermore it sheds light on the minimum venus flytrap domain (VFTD) and heptahelical domain (HHD) requirements for Ca\(^{2+}\)\(_o\)-induced Ca\(^{2+}\)\(_i\) mobilisation.

This thesis provides evidence that the CaSR contains molecular determinants for G-protein coupling and specificity in its intracellular domains. Residues F706 in iL-2 and L797 in iL-3 were required for G-protein coupling as HEK-293 cells expressing the mutants, F706A or L797A, had markedly impaired Ca\(^{2+}\)\(_o\)-induced IP\(_1\) accumulation, Ca\(^{2+}\)\(_i\) mobilisation, ERK1/2 phosphorylation and cAMP suppression, of up to 95%. Residue E803 in iL-3 and the C-tail residues 866-1078, were identified as molecular determinants of G\(_q/11\) selectivity. HEK-293 cells expressing the E803A mutant or the 866X truncation mutant had impaired IP\(_1\) accumulation, Ca\(^{2+}\)\(_i\) mobilisation and ERK1/2 phosphorylation, previously demonstrated to be downstream of G\(_q/11\). On the other hand, Ca\(^{2+}\)\(_o\)-induced suppression of cAMP levels, which is at least in part coupled to G\(_i/o\), was not affected.

This thesis also reports the development of a heterodimer expression system that utilises the GABA\(_B\) trafficking system. Chimeric receptors were constructed where the CaSR C-tail residues 876-1078 were replaced by the GABA\(_B1\) C-tail residues 854-960 to generate CaSR-
B1, or CaSR C-tail residues 909-1078 were replaced with residues 760-941 of the GABA_B2 C-tail to generate CaSR-B2. Appenage of the GABA_B1 C-tail after S875 was found to suppress surface expression due to the presence of an ER retention motif in the GABA_B1 C-tail, resulting in an absence of Ca^{2+}_o-induced Ca^{2+}_i mobilisation in CaSR-B1 expressing HEK-293 cells. Co-expression of CaSR-B1 with CaSR-B2 resulted in enhanced CaSR-B1 surface expression, which was dependent on the interaction of GABA_B1 and GABA_B2 C-tails.

Finally, this heterodimer trafficking system was used to study the minimum subunit requirements for nutrient sensing and Ca^{2+}_o-induced Ca^{2+}_i mobilisation. Introducing the VFTD inactivating mutations, G143E or R185Q, into one or both VFTDs demonstrated that a single, functional VFTD was sufficient to attain wild-type efficacy, but two functional VFTDs were required for wild-type Ca^{2+}_o potency. To investigate the HHD requirements for signalling, the F706A or L797A mutations that impair G-protein coupling, or the E803A mutation, which impairs G_q/11 coupled signalling, were introduced into the heterodimer trafficking system. The results demonstrate that two, functional HHDs are required for G-protein coupling, but a single, functional HHD is required for G-protein selectivity.
Abbreviations

1,25(OH)$_2$D$_3$  1,25-dihydroxyvitamin D$_3$

25(OH)D$_3$  25-hydroxyvitamin D$_3$

AA  Arachidonic acid

ADH  Autosomal dominant hypoparathyroidism

BRET  Bioluminescence resonance energy transfer

BSA  Bovine serum albumin

Ca$^{2+}_i$  Intracellular calcium ions

Ca$^{2+}_o$  Extracellular calcium ions

cAMP  Cyclic adenosine monophosphate

CaSR  Extracellular calcium-sensing receptor

CRD  Cysteine-rich domain

C-tail  Carboxyl terminus

DA  1,2 diacylglycerol

DMEM  Dulbecco's modified eagle medium

dNTP  Deoxy-nucleotide triphosphate

EC$_{50}$  Half maximal effective concentration
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<td>Extracellular domain</td>
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<tr>
<td>EGFR</td>
<td>Epithelial growth factor receptor</td>
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<tr>
<td>E&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximal response</td>
</tr>
<tr>
<td>EPAC</td>
<td>Exchange protein directly activated by cAMP</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ERGIC</td>
<td>ER golgi intermediate compartment</td>
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<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
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<td>HEK-293</td>
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<td>HRP</td>
<td>Horse-radish peroxidase</td>
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pH<sub>o</sub>  Extracellular pH

PI 3-K  Phosphatidylinositol 3-kinase

PI-PLC  Phosphatidylinositol phospholipase C

PKA  Protein kinase A

PKC  Protein kinase C

PLC  Phospholipase C

PLD  Phospholipase D

PSS  Physiological saline solution

PTH  Parathyroid hormone

PTHrP  Parathyroid hormone related peptide

RAMP  Receptor activity modifying proteins

RGS  Regulator of G-protein signalling

ROI  Region of interest

rpm  Revolutions per minute

SDM  Site-directed mutagenesis

SMG  S-methylglutathione

SRE  Serum response element

TAL  Thick ascending limb
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<tr>
<td>$T_m$</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VFTD</td>
<td>Venus flytrap domain</td>
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<td>WT</td>
<td>Wild-type</td>
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General
Introduction
Chapter 1  General Introduction

1.1 Physiological role of Ca\(^{2+}\) ions

Ca\(^{2+}\) ions are important physiological messengers, playing a role in a multitude of signalling pathways in the cytosol of eukaryotes. Their versatile chemical properties, including a favourable ionic radius and hydration status, allow them to bind to a wide range of intracellular protein targets. Basal cytosolic free Ca\(^{2+}\) concentrations are very low, ranging between 100-500 nM, whereas subcellular compartments may contain up to several hundred μM of free Ca\(^{2+}\). This difference allows Ca\(^{2+}\) ions to act as important secondary messengers [1, 2].

In recent decades, the role of extracellular ionised Ca\(^{2+}\) (Ca\(^{2+}_o\)) as a primary messenger has emerged [3]. Ca\(^{2+}_o\) regulates an array of cellular processes including secretion, proliferation, differentiation and apoptosis. This role is facilitated by the maintenance and sensing of differences in Ca\(^{2+}\) concentrations across the cell membrane [4].

1.2 Extracellular Ca\(^{2+}\) homeostasis

In the serum 35-40% of Ca\(^{2+}\) is complexed to proteins, while 10% is complexed to anions. The remaining 50-55% of Ca\(^{2+}\) is free, ionised Ca\(^{2+}\). Ca\(^{2+}_o\) levels are maintained within a narrow range of 1.1-1.3 mM and deviate by no more than 2% in healthy adults [5]. Deviations from the set point are detected by the parathyroid and thyroid glands instigating an interplay between parathyroid hormone (PTH), calcitonin (CT) 1,25-dihydroxyvitamin D\(_3\) (1,25(OH)\(_2\)D\(_3\)), the bones, kidney and gastro-intestinal tract to restore the Ca\(^{2+}_o\) concentration (Figure 1.1) [3, 6].
Figure 1.1 Extracellular calcium homeostasis

Serum Ca\(^{2+}\) levels are regulated by a network involving the parathyroid, kidney, bones and gastro-intestinal tract [7].

A rapid onset of hypocalcemia, or a drop in Ca\(^{2+}\) concentration, stimulates PTH secretion from the chief cells of the parathyroid. PTH promotes bone resorption and increased Ca\(^{2+}\) uptake by the kidneys. In prolonged hypocalcemia, PTH stimulates the production of \(1,25(OH)_2D_3\) from its precursor, 25-hydroxyvitamin D\(_3\) (25(OH)D\(_3\)), which, in addition to promoting the effects of PTH, acts to increase Ca\(^{2+}\) uptake from the intestinal tract. The net effect is an increase in Ca\(^{2+}\) concentration, which stimulates CT secretion from the thyroid.
gland to inhibit bone resorption and negatively feeds back to reduce PTH secretion [8]. This regulation mechanism needed a sensor to measure and respond to acute changes in $\text{Ca}^{2+}_o$ [6].

### 1.3 The extracellular $\text{Ca}^{2+}$-sensing receptor

Indirect evidence for a “calciostat” in parathyroid cells came by observing that increasing $\text{Ca}^{2+}_o$ led to inositol 1,4,5 triphosphate (IP$_3$) accumulation and intracellular $\text{Ca}^{2+}$ ($\text{Ca}^{2+}_i$) mobilisation in these cells [3]. The “calciostat” was identified after being cloned from a library of bovine parathyroid cDNA and named, the extracellular calcium-sensing receptor (CaSR) [9]. Subsequently, the CaSR was identified in a variety of human tissue including human adenomatosus parathyroid gland and parafollicular cells of the thyroid, as well as from tissues in species that possess parathyroid glands, such as birds, mammals and amphibians [10-12]. CaSR homologs have also been identified in species lacking parathyroid glands, like fish, suggesting that the evolution of CaSR preceded the evolution of parathyroid glands [11]. This view is supported by the cloning of a much smaller extracellular $\text{Ca}^{2+}_o$-sensing receptor in *Arabidopsis* that shares very limited sequence homology with the human CaSR [13].

In humans, the CaSR gene is encoded by seven exons located on the long arm of chromosome 3 [11]. The protein is 1078 amino acids long and is active at the cell surface as a dimer [10]. It is a member of the G-protein coupled receptor (GPCR) superfamily, specifically belonging to class C. Other members of class C include the metabotropic glutamate receptors (mGluRs), the GABA$_B$ receptors which respond to the neurotransmitter, $\gamma$-amino butyric acid (GABA), as well as other taste and pheromone receptors [11]. Although class C receptors share a very low level of sequence homology, homology is high at the topological level (Figure 1.2). Class C receptors are typified by a large, extracellular amino terminal domain that forms a bi-lobed structure called the venus flytrap domain (VFTD) and a connecting cysteine-rich domain (CRD). They also have the characteristic GPCR
transmembrane, heptahelical domain (HHD) and a large intracellular carboxyl terminus (C-tail) [10].

![Diagram of Class C GPCR topology]

**Figure 1.2 Class C GPCR topology**

Binding of agonists and/or positive allosteric modulators (PAM) in the VFTD or HHD of class C GPCRs allows intracellular proteins to interact with the HHD and C-tail domain in a way that allows modulation of different intracellular signalling pathways. Many class C GPCRs, including the CaSR, are functionally active at the cell surface as homodimers.

### 1.4 CaSR mutations and disease

Activating and inactivating mutations in the CaSR affect $\text{Ca}^{2+\text{o}}$ homeostasis in individuals which carry them [6]. Familial hypocalciuric hypercalcemia (FHH) arises from heterozygous loss-of-function mutations. These mutations increase the level of $\text{Ca}^{2+\text{o}}$ necessary for PTH secretion. As a result patients present with mild hypercalcemia, typically about 2.75 mM, with normal PTH levels and have reduced urinary $\text{Ca}^{2+}$ excretion [6, 14]. FHH patients are often asymptomatic and the condition is considered benign. The loss-of-function may arise from mutations which impair the function and/or plasma membrane expression of the CaSR [15].
Neonatal severe primary hyperparathyroidism (NSHPT) generally arises from inactivating mutations in both alleles of the CaSR gene [6]. The mutation is often a homozygous FHH mutation, but two different loss-of-function mutations in the CaSR gene have also been reported as well as a patient who harboured a single, heterozygous mutation in the CaSR gene [16]. The latter suggests that factors other than CaSR contribute to NSHPT.

NSHPT patients present within the first six months of life with a phenotype characterised by symptomatic moderate to severe hypercalcemia (3-7.7 mM), hyperparathyroidism and associated bony changes [6]. Bone changes are a result of undermineralisation resulting in developmental deformities and fractures. In its most severe form the condition is lethal and requires a total parathyroidectomy for treatment [6, 15].

Autosomal dominant hypoparathyroidism (ADH) is caused by gain-of-function mutations of the CaSR which lower the Ca\(^{2+}\) set-point for PTH secretion. Although the condition is typically asymptomatic, it is characterised by hypocalcemia, hypoparathyroidism and often hypercalciuria [6, 15].

### 1.5 Structural Domains of the CaSR

#### 1.5.1 Venus Flytrap Domain

The first 612 amino acids of the CaSR constitute the extracellular domain (ECD), which consists of the VFTD and the CRD [10]. A signal peptide consisting of the first 19 residues at the NH\(_2\)-terminal, is cleaved off in the functional protein [17]. The ECD contains 11 putative \(N\)-linked glycosylation sites. \(N\)-linked glycosylation of eight of these sites has been found to be important for CaSR maturation and normal cell surface expression [18, 19].
Figure 1.3 Model structure of the CaSR VFT domain high-lighting proposed Ca$^{2+}$- binding sites in the extracellular region of the protein

Site 1 (orange) located in the hinge of the VFT, is the conserved binding site for amino acids and may also bind Ca$^{2+}$. Site 2 (green) and site 3 (red) are considered to be moderate-affinity binding sites, while site 4 (blue) and site 5 (purple) are believed to have a relatively lower-binding affinity for Ca$^{2+}$ than the other sites [20].

The VFTD spans residues 20-536 [21]. The VFTD contains structural and sequence homology to the bacterial periplasmic binding proteins involved in nutrient sensing and uptake [22]. Although the CaSR VFTD structure is yet to be solved, based on the crystal structures of the homologous mGluR-1, mGluR-3 and mGluR-7, it is predicted to form a bilobed structure. The VFTD can interchange between the “open”, inactive conformation, and the “closed”, active form. The binding of agonists and/or positive allosteric modulators (PAMs) in the VFTD are believed to stabilise the “closed”, active conformation of the VFTD [23, 24].
A range of CaSR activators and PAMs bind in the VFTD, including $\text{Ca}^{2+}_o$, although binding sites are yet to be resolved [25]. To determine putative $\text{Ca}^{2+}_o$ binding sites, Huang et al. used a grafting approach and measured the $\text{Ca}^{2+}$ binding properties of small peptides from the CaSR introduced in a scaffolding protein unresponsive to $\text{Ca}^{2+}_o$ [26]. Their approach predicted five $\text{Ca}^{2+}_o$ binding sites in the CaSR (Figure 1.3). Studies with chimeric receptors have identified the VFTD as the site of interaction of other cationic activators including $\text{Mg}^{2+}$, $\text{Gd}^{3+}$ and neomycin [25].

The VFTD also interacts with PAMs, including L-amino acids and $\gamma$-glutamyl peptides [27, 28]. The double mutant T145A/S170T selectively disables enhancement of CaSR $\text{Ca}^{2+}_o$ sensing by L-Phe and S-methylglutathione (SMG) without impairing $\text{Ca}^{2+}_o$ signalling indicating a role of these residues in PAM binding. This is consistent with the proposed L-amino acid binding site (Site 1, Figure 1.3), consisting of S147, S170, D190, Y218 and E297, which is conserved in class C GPCRs [20, 26]. Interestingly, this site is also proposed to be a moderately high affinity $\text{Ca}^{2+}$ binding site, which maybe occupied by $\text{Ca}^{2+}$ at physiological $\text{Ca}^{2+}_o$ concentrations, but binding of amino acids may stabilise a conformation that facilitates binding of $\text{Ca}^{2+}_o$ to sites with a lower affinity [20].

Residues in the VFTD are important for CaSR dimerisation. Dimerisation is critical for signal transduction as the CaSR is functionally active on the cell surface as a homodimer [29, 30]. C129 and C131 participate in disulphide bonds across the dimer interface with the double mutant C129S/C131S failing to dimerise [31]. Intramolecular, non-covalent interactions between L112 and L156 also contribute to maintaining the CaSR dimer [32].
1.5.2 Cys-rich Domain

The CRD, spanning from residues 537-612 of the CaSR, is a highly conserved domain amongst class C GPCRs, with the GABA<sub>B</sub> receptors being a notable exception. It consists of nine highly conserved cysteine residues within a span of about 60 amino acids and connects the VFTD to the HHD (Figure 1.4). Deletion and mutagenesis studies of this domain point to a role in regulating CaSR function and cell surface expression [33, 34].

![Figure 1.4 Schematic representation of the cysteine-rich domain](image)

Nine cysteine residues, highly conserved amongst class C GPCRs, exist in this region and participate in disulphide bonds. Solid lines represent the three permanent intradomain disulphide bonds predicted to exist within the CRD. The bold broken line represents an intradomain disulphide bond which is hypothesised to rearrange upon receptor activation to form an inter-subunit disulphide bond. The fine broken line represents a potentially unstable interdomain disulphide predicted to exist with C236 in the VFTD.

Three permanently linked inter domain disulphide bonds are predicted to exist within the CRD [35, 36]. The mGluR-3 crystal structure showed a disulphide between the VFTD and the CRD thought to communicate ligand binding between the domains [36]. The cysteine residues from the mGluR-3 structure are conserved in the CaSR and correspond to C236 of the VFTD and C561 of the CRD [20].

A recent study of mGluR-2 also suggests that the proximity of cysteine residues in the CRD within a dimer may also be important for receptor activation [37].
Although no ligand binding sites in the CaSR CRD have been identified, the finding that the CRD of T1R3, a class C GPCR, binds the small, sweet tasting proteins, brazzein and thaumatin, raises the possibility that the CRD, in addition to being important for signal transmission, receptor expression and activation, may also be a common ligand interaction site in class C GPCRs [38, 39].

1.5.3 **Heptahelical Domain**

Hydropathy plot analysis of the cloned bovine CaSR showed it possessed the characteristic GPCR HHD consisting of seven α-helices spanning the cell membrane linked to each other by three intracellular and three extracellular loops and included residues 613-862 (Figure 1.5) [9].

The “headless” receptor, which retained sensitivity to cationic activators of the CaSR despite lacking the CaSR extracellular domain, demonstrated ligands also bind in the CaSR HHD [40]. Furthermore calcimimetics and calcilytics, such as Cinacalcet and NPS-2143, respectively, also bind in the HHD with mutational analysis suggesting they have overlapping but different sites [40, 41]. F668A, F684A and E837I mutants attenuated responses to both the calcimimetic and calcilytic, however the R680A only impaired the calcilytic response, retaining sensitivity to calcimimetics [41].

The intracellular loops of the CaSR couple to intracellular proteins to initiate signalling pathways. Mutational analysis have highlighted the role of F707 and L704, in iL-2, and L798, E802 and E804 in iL-3 of the bovine CaSR in coupling efficiently to phospholipase C [42]. Residues in the intracellular loops also play a role in mediating CaSR expression, as mutating E804 impaired surface expression [42].
Acidic residues in extracellular loops maintain the CaSR in the inactive conformation [43]. Mutating D758, E759 and E767 increased the CaSR’s sensitivity for Ca$^{2+}$o.

*Figure 1.5 Schematic representation of the CaSR HHD*

The HHD consists of seven α-helices (white circles), three extracellular loops (light grey circles) and three intracellular loops (dark grey circles). Residues interacting with both calcimimetics and calcilytics are enclosed in double-lined circles, while those interacting with just calcilytics are enclosed in a single, bold circle. Black residues are required for maintaining the CaSR in its inactive conformation. Residues enclosed in broken-lined circles are involved in PI-PLC signalling.

### 1.5.4 Carboxyl terminus

The CaSR’s carboxyl terminus (C-tail) extends from residue 863-1078 (Figure 1.6) [44]. Deletion of the CaSR C-tail does not abolish receptor expression at the cell surface, even
though the receptor response is eliminated [45, 46]. In particular deletion of the C-tail abolished Ca$^{2+}$o mediated phospholipase C (PLC) signalling [46].

Mutational analysis has revealed residues between 874-888 to be important for CaSR signalling [47]. In particular, T888 appears to be the primary substrate for protein kinase C (PKC) phosphorylation and a calyculin-sensitive phosphatase dephosphorylation, which desensitises and resensitizes the CaSR to Ca$^{2+}$o, respectively [48, 49]. The C-tail also has two more PKC phosphorylation sites at S895 and S915 [49]. Changes in the rate of Ca$^{2+}$o-induced phosphorylation and dephosphorylation are the cause of Ca$^{2+}$i oscillations and sustained Ca$^{2+}$i concentrations [50]. Additionally, residues 868-886 are important for co-operativity [45].

This intracellular domain also contains elements that affect agonist-independent receptor expression by regulating receptor internalisation, recycling to the cell surface and targeting for lysosomal degradation [51]. Deletion of residues 920-970 of the C-tail generated a functional receptor that was expressed at higher levels at the cell surface, recycled to the cell membrane more rapidly upon internalisation and, unlike the WT CaSR, was prevented from lysosomal degradation [46, 51].

The C-tail is also involved in interactions with other proteins. The scaffolding protein, Filamin, interacts with the CaSR at residues 907-997 and is important for CaSR-mediated ERK signalling [52]. The adaptor protein, 14-3-3, has also recently been identified as a partner that binds to the proximal region of the C-tail and promotes ER retention of the CaSR as well as modulating Rho signalling [53, 54]. Studies on the mGluR-1 receptor have also revealed a role of the C-terminal in G-protein coupling [55].
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Figure 1.6 The CaSR C-tail

The 216 residues of the CaSR C-tail play an important role in receptor activation (double-lined circles), expression (grey circles), PKC phosphorylation (▼), co-operativity and de-sensitisation (broken line). They also interact with partner proteins such as Filamin. The high-affinity Filamin binding site is shown as black residues.

1.6 CaSR Activators and Ligands

The CaSR has a reputation of being a promiscuous receptor that is able to bind a diverse range of ligands, both physiological and synthetic, in its multiple binding sites, thereby allowing it to function as an integrator of multiple metabolic signals (Figure 1.7) [56].
In addition to its primary physiological agonist, \( \text{Ca}^{2+} \), the CaSR is able to interact with a variety of ligands of different sizes and chemical structures. Spermine, an organic polyvalent cation, may act as an agonist or a PAM. The phenylalkylamine, Cinacalcet, acts as a PAM as do L-amino acids, like L-Phe, and \( \gamma \)-glutamyl peptides like S-methylglutathione.
1.6.1 Type I Calcimimetics

Ca\(^{2+}\) is the principal physiological agonist of the CaSR, however it has a relatively low potency with a half maximal effective concentration (EC\(_{50}\)) in the millimolar range \([9, 57]\). The CaSR’s sensitivity to small changes in Ca\(^{2+}\) arises from the high co-operativity of multiple Ca\(^{2+}\) binding sites in the CaSR with a hill coefficient of ~3-4 \([56]\).

Type I calcimimetics are organic and inorganic divalent, trivalent and polyvalent cations that are agonists of the CaSR \([9, 58]\). In general, the concentration of a cation required to activate the CaSR varies inversely with its charge. A multitude of inorganic cations have been identified as activators of the CaSR, with trivalent cations like Gd\(^{3+}\), Tb\(^{3+}\) and Eu\(^{3+}\) being the most potent, activating the CaSR at sub-micromolar and micromolar concentrations ranging from 0.9-5 \(\mu\)M \([9, 59]\). Some divalent cations are also able to activate the CaSR at micromolar concentrations ranging from 100-700 \(\mu\)M, and include Fe\(^{2+}\), Co\(^{2+}\), Cd\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\) and Pb\(^{2+}\) \([59]\). Other divalent cations, like Mg\(^{2+}\), Ba\(^{2+}\), Sr\(^{2+}\) and Mn\(^{2+}\) activate at millimolar concentrations in the range of 2-7 mM \([56]\).

1.6.2 Polyamines

Polyamines are organic polycations produced \textit{in vivo} which are able to activate the CaSR \([60]\). Their potency seems to correlate well with the charge of a molecule, with spermine, possessing four positive charges, being more potent that spermidine which has three positive charges. Putrescine, which has two positive charges, has no effect on the CaSR even at high millimolar concentrations \([56, 60]\).

Spermine may behave as a PAM or an agonist of the CaSR. In the presence of 0.5 mM Ca\(^{2+}\) it is able to activate the CaSR at an EC\(_{50}\) of 500 \(\mu\)M. At higher Ca\(^{2+}\) concentration, spermine
can activate the CaSR at low micromolar concentrations [57, 60]. Due to the positive co-operativity with which spermine interacts with the CaSR, multiple spermine binding sites in the CaSR have been proposed [56, 60].

Nephrotoxic aminoglycosidic antibiotics are also known activators of the CaSR [9, 61]. Their potency correlates well with the number of amino groups on a molecule. Neomycin, which has six amino groups, activates the CaSR with an EC$_{50}$ of 77 μM while, tobramycin and gentamicin, both possessing five amino groups each, activate at higher micromolar concentrations. Kanamycin, with four amino groups, is effective at concentrations greater close to 3 mM [62]. The sensitivity of the CaSR to aminoglycosidic antibiotics is dependent on the extracellular pH (pH$_o$), which effects charge. Reducing the pH$_o$ was found to increase the CaSR’s sensitivity to tobramycin [62]. Furthermore, the nephrotoxic effects of aminoglycosidic antibiotics in the renal tubule are thought to be exerted via aminoglycosidic antibiotic induced activation of the CaSR [61].

1.6.3 Type II Calcimimetics

Type II calcimetics are synthetic compounds that work as PAMs to increase the CaSR’s sensitivity to Ca$^{2+}_o$. Phenylalkylamines are a class of type II calcimimetics that are based on the structure of Fendiline [58]. The first phenylalkylamine derivatives to be tested were NPS-568 and NPS-467. They are stereoselective activators with R-enantiomers being more potent than S-enantiomers. Furthermore, NPS-568 and NPS-467 both require the presence of a low level of Ca$^{2+}_o$ for CaSR agonism, confirming that they are PAMs rather than agonists of the CaSR [58]. Calcimetics are potential therapeutic agents for treating CaSR inactivating disorders. Indeed, NPS R-568 was successfully able to lower plasma levels of PTH in
patients with primary and secondary hyperparathyroidism, however was unsuitable because of a variable metabolic profile between individuals [63].

The second generation of phenylalkylamine derivatives acting on the CaSR have gained considerable therapeutic value with Cinacalcet HCl, an analog of NPS R-568, being approved for treatment of hypercalcemia in patients with parathyroid cancer and severe hyperparathyroidism in patients with chronic kidney disease [64].

1.6.4 Amino acids and peptides

The observation that amino acids in the presence of submaximal concentrations of Ca$^{2+}_o$ lead to Ca$^{2+}_i$ mobilisation in vitro confirmed that amino acids are PAMs of the CaSR [65]. The effect, as with phenylalkylamine derivatives, was stereoselective with L-enantiomers being more potent than D-enantiomers [65]. Furthermore, potency, which was in the millimolar range, varied according to the amino acid side chain with aromatic amino acids, phenylalanine and tryptophan, being most potent. Charged amino acids and branched-chain amino acids had little or no effect, respectively.

The γ-glutamyl tripeptides, glutathione and S-methylglutathione, are PAMs of the CaSR [27, 66]. In HEK-293 cells they enhanced Ca$^{2+}_i$ mobilisation and reduced cyclic adenosine monophosphate (cAMP) levels. Furthermore, they were able to inhibit PTH secretion in human parathyroid cells [27]. L-amino acid and tripeptide Ca$^{2+}_o$-sensing enhancement were disabled by the T145A/S170T double mutant, suggesting that γ-glutamyl peptides and L-amino acids interact and activate the CaSR in a similar way [27, 28].
1.6.5 Calcilytics

Calcilytics are negative allosteric modulators (NAMs) of the CaSR, blocking CaSR activity by reducing its sensitivity and/or maximal response to Ca$^{2+}$ [67-69]. The first such molecule to be synthesised was NPS-2143 which was able to inhibit Ca$^{2+}$ mobilisation by Ca$^{2+}$o and NPS R-467 in HEK-293 cells, and stimulate PTH secretion in bovine parathyroid cells [67]. Despite the development of other calcilytics including Calhex-231, NPS-2143 is the best defined calcilytic in terms of its pharmacology and therapeutic potential [68]

Calcilytics have potential as drugs with osteoanabolic/osteoblastic activity for the treatment of osteoporosis, provided they do not lead to prolonged increases in serum PTH levels which may cause unwanted osteocatobolic effects (Figure 1.8) [68]. NPS-2143 increased bone turnover in osteopenic rats when administered orally, which however, was balanced by the osteocatobolic activity of high, sustained PTH levels leading to no net change in bone mineral density [70]. Recently, two new calcilytics, SB-423562 and ATF936, were both able to increase bone mineral density in osteopenic rats as they led to transient increases in PTH levels, preventing bone loss [71, 72]. The effects of both drugs on PTH levels were reproduced in humans with no adverse effects, making them candidates for CaSR-mediated treatment of osteoporosis.

1.6.6 Other activators

CaSR activation has been shown to be sensitive to ionic strength, suggesting it may play a physiological role in sensing ionic strength [73]. Increasing the ionic strength with NaCl and other salts reduced the CaSR’s sensitivity to Ca$^{2+}$o, while reducing the ionic strength activated the CaSR at lower Ca$^{2+}$o.
The CaSR has also been proposed as a sensor of physiological pH [74]. The CaSR’s sensitivity for its physiological agonists increased and decreased with extracellular pH.

Figure 1.8 Clinically tested calcilytics
Calcilytics have therapeutic potential in the treatment of osteoporosis due to their osteoanabolic activity. NPS-2413 was found to have unwanted osteocatabolic effects in clinical studies having no net effect on bone. Two new calcilytics, SB-423557 and ATF936 however have been found to increase net bone mineral density in osteopenic rats. Their effect on bone in humans is yet to be evaluated.
1.7 Requirements for CaSR function

Intracellular signalling by the CaSR requires that the CaSR is in a state where it can be activated. The CaSR’s requirements for dimerisation, glycosylation, receptor trafficking and density need to be met. Once expressed at the cell surface as a homodimer with a sufficient receptor population, the CaSR gains its functional capacity [11, 19, 75].

1.7.1 Dimerisation

For functional activity, the CaSR must reside at the cell surface as a homodimer, held together by covalent and non-covalent interactions in the VFTD [30, 32]. Dimerisation occurs in the endoplasmic reticulum (ER) prior to and may influence mature glycosylation [76]. Although it is important for trafficking of the receptors to the cell surface, it is not a prerequisite [77].

Dimerisation is important for a number of essential CaSR signalling properties. Perhaps most importantly, dimerisation allows the CaSR to respond to small changes in high concentrations of $\text{Ca}^{2+}$ by increasing the number of $\text{Ca}^{2+}$ binding sites allowing for greater co-operativity than a monomeric receptor [25].

Dimerisation may also aid in masking the effect of mutations [11]. Bai et al. (1998) were able to show heterodimerisation of WTCaSR with the non-functional A877 truncated CaSR [30]. They utilised this heterodimerisation system to test the effect of mutations which in CaSR homodimers impaired signalling [78]. Co-expression of the null G143E mutant with the A877X receptor lead to a partial recovery in CaSR function, however the single intact VFTD was not sufficient for wild-type activity [78]. This demonstrated that the CaSR dimer has two functionally separable monomer subunits each of which contributes to CaSR-mediated
signalling. This seems to be common to class C GPCRs as confirmed by the observation that the two closed VFTDs are required for full activity in mGluR-5 [79]. Partial activity by mGluR-5 was observed when one of the two extracellular agonist binding domains was impaired.

CaSR cell surface expression and trafficking are promoted by dimerisation. The CaSR’s C-tail contains an α-helical domain between residues 877-891 as well as a putative ER retention sequence from Arg896-Arg898. Homodimerisation leads to masking of the ER retention sequence by the α-helix, promoting cell surface expression [46, 76].

The CaSR has also been isolated as a heterodimer with GABA\textsubscript{B} receptors in HEK-293 cells and hippocampal neurons [76]. CaSR function and expression decreased or increased depending on whether it was co-expressed with GABA\textsubscript{B1} or GABA\textsubscript{B2}, respectively. This finding prompts the idea of dimerisation also allowing the CaSR to vary its ligand sensing, G-protein coupling and signalling upon heterodimerisation with other class C GPCRs.

1.7.2 Receptor trafficking and cell surface expression

Trafficking of the nascent CaSR polypeptide from ribosomes to the plasma membrane for expression are dependent on protein interactions, the maturation status of the receptor, and receptor activation [11, 20, 54, 80].

The 19 amino acid N-terminal signal peptide commences the trafficking process of the nascent CaSR polypeptide from ribosomes to the ER lumen. Two naturally occurring mutations in this peptide, L11S and L13P, impaired cell surface expression as the translated peptide failed to progress to the ER lumen from the ribosomes [81]. The un-glycosylated CaSR monomer appears as a 120 kDa band on western blots.
The ER is where glycosylation of the CaSR commences [18, 19]. Mannose moieties are added to asparagines residues that enter the ER lumen and are necessary for release from the ER [54, 77, 82]. This immaturely glycosylated high mannose form of the CaSR is not expressed at the cell surface and the monomer appears as a 140 kDa band on western blots. CaSR dimerisation also occurs in the ER [77]. The role of CaSR dimerisation as a protein-protein interaction that is important for cell surface expression has been mentioned above.

The ER also serves as a checkpoint of CaSR biosynthesis [83]. Some loss of function mutants associated with FHH and NSHPT were sent for degradation from either the ER or the ER-golgi intermediate compartment (ERGIC) due to poor protein folding and/or function [83].

The correctly-folded, immaturely glycosylated and dimerised receptor is then trafficked from the ER to the Golgi [77]. In HEK-293s, the C-tail interacts with p24A, a member of the cargo receptor family, providing stability for the immature CaSR as it is secreted form the ER and is trafficked to the Golgi [84]. Overexpression of p24A increased total and cell surface CaSR expression. In contrast, expression of a defective p24A mutant increased CaSR retention in the ER and degradation.

Another important interaction which promotes CaSR trafficking from the ER to the Golgi apparatus is with receptor activity modifying proteins (RAMPs) [80, 85]. In COS-7 cells lacking endogenous expression of RAMPs, the CaSR was localised to the ER. Coexpression with RAMP-1 or RAMP-3 promoted cell surface expression of the CaSR. Furthermore, silencing of RAMP-1 in HEK-293 cells, where RAMP-1 is endogenously expressed, impaired CaSR cell surface expression [85].

Mature glycosylation of the CaSR takes place at the Golgi [77]. Maturation involves the addition of complex carbohydrates to selected asparagines residues in the ECD [30] The N-
linked glycosylation status of the mature CaSR is important for cell surface trafficking [11, 86]. Removal of any 3-5 of the 8 N-linked glycosylation sites in the CaSR VFTD significantly impaired receptor expression as did tunicamycin treatment [18, 19]. The mature, fully glycosylated form of the CaSR expressed at the cell surface appears as a ~160 kDa band on western blot analysis [86].

The Golgi, like the ER can also serve as a biosynthetic quality control checkpoint as some loss of function mutants associated with FHH/NSHPT were transported to the Golgi, as evidenced by furin cleavage, however were not present at the plasma membrane [83]. These mutants are likely sent for proteosomal or lysosomal mediated degradation after trafficking from the Golgi.

### 1.7.3 Cell surface receptor density

The level of cell surface expression of the CaSR correlates with CaSR function [11]. Mutations and treatments of the CaSR which reduce or impair CaSR expression at the cell surface also inhibit CaSR function, for example the R227Q mutant and tunicamycin treatment [19, 77, 83].

Net receptor density is a result of equilibrium between receptor trafficking to the cell surface, internalisation, recycling and degradation. Furthermore, interactions of the CaSR with proteins and ligands are involved in regulating receptor density.

In a recent study by Grant et al. (2011), CaSR expression at the cell surface was found to be regulated by receptor activation. A basal level of CaSR expression was found at the cell surface at all times. Activation of these receptors by elevated Ca\(^{2+}\) promoted trafficking of intracellular pools of CaSR to the plasma membrane where they participated in signalling.
Receptors from the cell surface were endocytosed and most of them were sent for lysosomal degradation rather than recycled back to the cell surface. The net increase in plasma membrane CaSR expression resulted in enhanced intracellular signalling [54].

The activity of some loss of function mutants associated with FHH and NSHPT, which are expressed poorly at the cell surface can be rescued by the chaperoning effects of the calcimimetic, NPS R-568, or treatment with the proteasome inhibitor, MG132 [83, 87]. In the case of NPS R-568 the functional rescue is brought about by increasing the number of the receptors at the cell surface and also by increasing the sensitivity of cell membrane receptors to Ca\(^{2+}\). In the case of MG132, functional rescue is brought about by inhibiting proteosomal degradation of CaSR [83, 87].

The CaSR also undergoes lysosomal degradation due to a lysosomal targeting sequence scattered in the CaSR’s C-tail between resides 920-970 [51]. Deletion of this region of the C-tail increased plasma membrane receptor expression by preventing lysosomal degradation and enhanced recycling of the receptor to the cell surface after internalisation.

Filamin is a scaffolding protein which promotes CaSR-mediated signalling when present in CaSR-expressing cells [52]. It interacts with the CaSR C-tail, to prevent degradation of the CaSR, promoting cell surface expression which has the net effect of enhancing CaSR-mediated signalling [88]. Furthermore, Filamin, as a scaffolding protein, is thought to encourage the correct spatial assembly of downstream cytoplasmic signalling proteins, thereby also enhancing the CaSR’s signalling capacity [52, 89].

CaSR degradation from the ER occurs via dorfin-mediated ubiquitination of lysine residues in the CaSR C-tail and intracellular loops, followed by proteasomal degradation. The interaction between CaSR C-tail is involved in the interaction with dorfin [90].
Residues 890-901 in the CaSR C-tail interact with 14-3-3 [54]. This interaction is responsible for intracellular retention of the CaSR. Truncation after Thr868 abolished the interaction.

1.8 CaSR-mediated signalling

Once the CaSR is functionally active at the cell surface, a process of ligand binding/sensing in the VFTD and/or HHD follows. Transmission of ligand interaction to downstream effector domains of the CaSR, namely the HHD and C-tail, occurs which then interact with intracellular proteins to initiate signalling cascades able to modulate the cell’s response to the critical change in extracellular ligand that activated the CaSR in the first place [44]. This process may occur over a time period of seconds to minutes [91]. Although elucidation of CaSR-mediated signalling has involved studies of the CaSR, much of the current understanding is based on translating research from related receptors, such as the mGlu receptors.

1.8.1 Ligand interactions

The initial step in CaSR signalling requires interactions with its ligands. Ligand interaction takes place at either the VFTD or the HHD [24, 92]. Although the precise mechanism by which CaSR agonists interact with their receptor is unknown, resolution of the mGluR-1 VFTD crystal structures in the unligated and ligated forms provided insight into receptor-ligand interaction of class C GPCRs in general [24, 92]. The disulphide linked VFTDs of mGluR-1 were found to exist in an open, unligated state (Figure 1.9). Binding of an antagonist stabilised the open conformation [92]. The binding of glutamate to one VFTD in the dimer via hydrogen bond interactions with residues in the binding pocket, led to a closure
of the VFTD and stabilisation of this conformation. The relative orientation of the VFTDs also changed from being 140° apart to being 70° apart upon glutamate binding. Kniazeff et al., have shown that while the closure of a single VFTD is necessary for receptor activity, the closure of both VFTDs is needed for full receptor activity [79].

![Figure 1.9 Ligand induced conformational changes in mGluR-1](image)

(A) The two VFTDs, represented in either darker or lighter colouring, of the mGluR-1 dimer are shown in the open, free form. Lobe 1 (blue) in each VFTD is separated from lobe 2 (red). (B) Binding of glutamate (yellow) leads to closure of the VFTD allowing lobe 1 and lobe 2 to come closer together. (C) The two VFTDs also structurally rearrange and reorient themselves relative to one another, such that in the free form they are 140° apart, whilst they are only 70° apart in the complexed form [24].

Ligand-induced conformational change was confirmed in fluorescence resonance energy transfer (FRET) experiments where the presence of glutamate changed the FRET efficiency between YFP or CFP-labelled mGluR-1 [93]. Additionally, the changes in FRET efficiency in fluorescently-labelled mGluR-1 were activator-dependent, with Gd³⁺ inducing a different FRET response to glutamate in the receptors [94]. Matsushita et al. showed in GABA_B receptors that the conformational change was largely intermolecular, with minimal change in intramolecular FRET efficiency in the presence of the principal activator, GABA [95].

Although a bioluminescence resonance energy transfer (BRET)-based approach was used to elucidate ligand-induced conformational changes in the CaSR, no change in BRET efficiency was observed, likely due to a suboptimal placement of acceptor and donor [96].
The intrasubunit and intersubunit rearrangement in response to ligand binding was hypothesised to be transmitted to the HHD via the CRD, leading to the recruitment of G-proteins by the intracellular regions of the CaSR [24]. Furthermore, selective signalling was hypothesised to arise from unique conformational changes induced by different receptor agonists.

1.8.2 Receptor activation

The presence of a disulphide bond linking the CRD and VFTD in the mGluR-3 crystal structure led to the proposal of an activation mechanism where ligand induced conformational changes in the VFTD of class C GPCRs was thought to be transmitted to the HHD via the CRD [36]. Indeed, mutating residue C234 in the VFTD of mGluR-5, proposed to be involved in the interdomain disulphide, led to uncoupling of the VFTD from the HHD preventing receptor activation [97].

To investigate the presence of an intradomain disulphide in the CaSR, Hu et al. inserted a tobacco etch virus (TEV) protease cleavage site between the VFTD and CRD. Following TEV protease digestion they were unable to detect any disulphide linked protein on a western blot, concluding the VFTD and CRD are not linked by a disulphide bond [98].

Nevertheless, the role of the CRD in CaSR signal transmission is important as mutations within a small 14 amino acid linker region in the CRD, common to all class C GPCRs except the GABA\textsubscript{B} receptors, were able to either activate the receptor, impair receptor activation or uncouple the VFTD from the HHD [34].

In addition to an interdomain disulphide bond within a subunit involved in signal transmission, an intersubunit disulphide bond between the two CRDs in a dimer has been
proposed to exist in the mGluR-2 [37]. Mutating C500 of mGluR-2, which is hypothesised to form an intradomain disulphide bond with C519, led to a constitutively active receptor. The constitutive activity of the C500A mutant is believed to arise from a permanent disulphide bridge between C519 of the two CRDs. It is hypothesised that in the wild-type receptor this intersubunit proximity is important for allosteric coupling of the VFTD to the HHD [37].

Intersubunit domain rearrangement also plays an important role in receptor activation [99]. Ligand binding in only one functional VFTD of mGluR-5 led to signalling from both HHDs. This showed that ligand binding in one VFTD not only activated the adjacent HHD in a *cis* manner, but was also able to activate the HHD of the other subunit in a *trans* manner as a result of intersubunit rearrangements [99].

1.8.3 *CaSR*-mediated signalling

Receptor activation in response to changes in extracellular ligands allow the CaSR to interact with various G-proteins [100]. G-proteins are composed of three subunits, α, β and γ, and exist as heterotrimers in the inactive state with GTP attached to the Gα. G-protein activation by upstream receptors, like the CaSR, involves an exchange of GTP for GDP on the α-subunit triggering the dissociation of the Gα subunit from the βγ subunits, both of which are then able to activate downstream signalling pathways [101]. The signalling pathways are then able to modulate the cells physiological response to the extracellular stimulus.

The CaSR is a pleiotropic GPCR that is able to couple to many G-proteins including Gq/11, G12/13 and Gi/o in a variety of cells [68]. G-protein activation allows the CaSR to induce changes in a variety of intracellular signalling pathways including those involving phospholipases, protein kinases, cAMP, Akt and Rho [102].
1.8.4 Phospholipase activation

Phospholipases (PL) reside in the cytosolic side of the plasma membrane, where upon activation by extracellular signals they are able to cleave phospholipids which act as intracellular signalling mediators [82]. The CaSR is able to activate phospholipases C, A₂ and D in response to elevated Ca²⁺₀ [103].

PLC activation in HEK-293 cells is pertussis toxin insensitive, suggesting it is regulated by Gᵣ₁₁ [4]. CaSR- mediated PLC activation is responsible for lipid hydrolysis leading to the generation of inositol triphosphate (IP₃) and 1,2 diacylglycerol (DA). IP₃ increases the rate of passive efflux of Ca²⁺ from intracellular stores, like the ER, increasing cytosolic Ca²⁺ (Ca²⁺ᵢ) [104]. DA stimulates protein kinase C (PKC), which is a negative regulator of Ca²⁺ release from intracellular stores as well as a stimulator of Ca²⁺ efflux from the cell [68, 104]. This leads to oscillations in Ca²⁺ᵢ levels in response to Ca²⁺₀, the frequency and pattern of which are important for signal transduction by the CaSR [105]. Sarcoplasmic/endoplasmic reticulum ATPases contribute to the CaSR-mediated Ca²⁺ᵢ mobilisation response as thapsigargin eliminated Ca²⁺ᵢ oscillations [106].

PKC plays an important role in CaSR-mediated Ca²⁺ᵢ oscillations as PKC inhibition impaired oscillations in the Ca²⁺ᵢ signal, while the PKC activator, phorbol 12,13-dibutyrate, abolished oscillations [105]. PKC exerts a negative feedback effect on Ca²⁺ᵢ levels by impairing CaSR activity via phosphorylation at T888, the primary CaSR PKC phosphorylation site. The T888A mutant impaired Ca²⁺ᵢ oscillations similar to PKC inhibitors [105]. In CaSR expressing HEK-293 cells, increasing CaSR activity in response to increasing Ca²⁺₀ stimulates protein phosphatase 2A activity, which dephosphorylates the T888 residue, leading to the observed sustained increase in Ca²⁺ᵢ levels [50]. Thus, CaSR-mediated Ca²⁺ᵢ
oscillations are thought to arise by dynamic phosphorylation and de-phosphorylation of the CaSR, the rates of which determine the frequency and pattern of Ca\(^{2+}\) oscillations [50].

Activation of PLA\(_2\) leads to an accumulation of arachidonic acid (AA) in cells [103]. PLA\(_2\) stimulated AA release lies downstream of G\(_q\) and PLC activation and is regulated by PKC as it was insensitive to pertussis toxin, was impaired by the PLC inhibitor U73122 and PKC inhibition reduced AA release [103, 107].

The CaSR also activates PLD leading to phosphatidic acid generation in CaSR expressing HEK-293 cells and MDCK cells [100, 103]. PLD activity arises from coupling of the CaSR to G\(_{12/13}\), which activates the Rho family of G-proteins. PLD activation provides a link as to how the CaSR may regulate the cytoskeleton, although this has yet to be confirmed [108].

1.8.5 Protein Kinase mediated signalling

Mitogen-activated protein kinases (MAPKs) regulate cellular activities by regulating the phosphorylation status of cytosolic proteins or nuclear proteins, which in turn regulates the activity of the proteins or leads to gene transcription [109]. The activation of the protein kinases c-SRC, ERK1 and MAPK in response to elevated Ca\(^{2+}\) was first established in rat fibroblasts. The activation was mediated by CaSR as CaSR agonists stimulated activity of the protein kinases while expression of the null mutant, R796W, inhibited protein kinase activity [110].

ERK1/2 phosphorylation is enhanced by CaSR mediated signalling of Ca\(^{2+}\) fluctuations, although the signalling pathways involved in this are complex and have not been completely resolved [100]. In HEK-293 cells stably expressing CaSR (HEK-CaSR), but not HEK-293 cells, ERK1/2 phosphorylation was stimulated by the calcimimetic NPS R-467, but not NPS S-
467, in addition to Ca\(^{2+}\) [109]. That ERK1/2 phosphorylation was inhibited by pre-treatment with pertussis toxin, suggesting the pathway was G\(_i\) mediated. However, G\(_q\) also plays a role in CaSR-mediated ERK1/2 phosphorylation as inhibition of PI-PLC and PKC inhibited ERK1/2 phosphorylation while activation of PKC stimulated ERK1/2 phosphorylation.

ERK1/2 activation by CaSR in HEK-CaSR cells involves a triple membrane spanning signalling mechanism whereby the CaSR is able to transactivate the epithelial growth factor receptor (EGFR) via activation of an extracellular matrix metalloproteinase, promoting release of the EGFR peptide which binds to EGFR leading to ERK1/2 phosphorylation [68, 111]. Filamin-A interaction with the CaSR C-tail is also required for ERK1/2 activation [112]. Phosphatidylinositol 3-kinase (PI 3-K) has also been implicated in CaSR-mediated ERK1/2 phosphorylation in HEK-CaSR, but not Rat-1 fibroblast cells, demonstrating cell specific signalling by the CaSR [100, 110, 113].

In addition to ERK1/2, the CaSR is able to stimulate MEK1/2 and p38 MAP kinase phosphorylation in HEK-CaSR cells, resulting in increased parathyroid hormone-related protein (PTHrP) secretion [114].

1.8.6 cAMP inhibition

The CaSR is able to regulate levels of the small, intracellular metabolite, cyclic AMP (cAMP) by coupling to the inhibitory G-protein, G\(_{i/o}\) or the stimulatory G-protein, G\(_s\). G\(_{i/o}\) inhibits the activity of adenylyl cyclase, thereby reducing cAMP levels, while G\(_s\) stimulates adenylyl cyclase activity leading to a corresponding rise in cAMP levels [82, 102]. Prior to the cloning of the CaSR, Chen et al., had shown that bovine parathyroid cells responded to a range of divalent cations, including Ca\(^{2+}\), by a reduction in cAMP levels [115]. The reduction
in cAMP levels in response to high Ca\textsuperscript{2+} and the CaSR modulator NPS R-467 was also observed in HEK-CaSR cells [116].

CaSR-mediated cAMP regulation may play a role in the pathogenesis of breast cancer [117]. In the normal breast cancer cell line, MMEC, CaSR activation inhibited parathyroid hormone related peptide PTHrP secretion by coupling to G\textsubscript{i}. In the malignant breast cancer cell lines, Comma-D and MCF-7, CaSR activation resulted in increased PTHrP secretion by coupling to G\textsubscript{s}. This G-protein coupling switch by the CaSR maybe an underlying mechanism of disease, as increased PTHrP secretion effects normal bone and calcium metabolism [117].

1.8.7 Akt signalling

The CaSR is able to activate phosphatidylinositol 3-kinase (PI 3-K) leading to phosphorylation and activation of Akt [118-120]. In the H-500 rat cancer cell line, Ca\textsuperscript{2+} activation of CaSR induced P1 3-K activation, which had anti-apoptotic and proliferative effects [118]. Ca\textsuperscript{2+} was also able to induce CaSR-mediated Akt phosphorylation in colonic myofibroblasts. Aminoglycosidic antibiotic activation of CaSR led to Akt phosphorylation in HEK-293 cells [119].

1.8.8 Rho mediated signalling

The CaSR stimulates the serum response element (SRE) upon activation by a G\textsubscript{q} coupled pathway in HEK-293 cells [89]. This activation is dependent on Rho. The Rho A signalling pathway requires filamin A to generate the cystoskeletal scaffold necessary for CaSR-mediated Rho signalling. 14-3-3 proteins also regulate CaSR-mediated Rho signalling [53]. Over expression of the 14-3-3\textsubscript{θ} isoform attenuated Rho signalling [53].
The pleiotropic CaSR couples to $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ to induce changes in a variety of intracellular metabolites (dashed outline) including ERK, cAMP and $Ca^{2+}$, which may be assayed for. CaSR activators which stimulate each of the pathways have been listed above.
1.9 CaSR tissue distribution & function

The CaSR is expressed widely in tissues of the human body. CaSR coupling to G-proteins and other downstream effector proteins in response to its extracellular activators and modulators is tissue and cell-type specific, as are the effects of CaSR signalling [121]. In addition to its roles in the parathyroid and thyroid, discussed in section 1.2 and 1.3, the CaSR functions in both normal and diseased states, and therefore is a potential therapeutic target.

Figure 1.11 CaSR expression and function in the human body

The CaSR is widely expressed in the human body performing a diverse array of functions.
1.9.1 Kidney

In the kidney the CaSR plays an important role in the renal regulation of not only $\text{Ca}^{2+}_o$ homeostasis, but also cation transport, urine concentration and acidification, and renin secretion [122]. CaSR expression occurs in different parts of the kidney nephron and the function of the CaSR differs accordingly.

In proximal tubule cells, the CaSR is expressed on the apical membrane [123]. Here variations in CaSR expression regulate $\text{Ca}^{2+}$ and inorganic phosphate ($\text{P}_i$) reabsorption and urinary excretion in a PTH dependent and independent manner. CaSR expression levels vary inversely with dietary phosphate intake in this region [124]. The CaSR also plays a role in regulating the calcemic effects of $1,25(\text{OH})_2\text{D}_3$ on the intestinal uptake and urinary excretion of $\text{Ca}^{2+}$ [122, 125]. In mice lacking the CaSR, an enhancement in the calcemic effects of $1,25(\text{OH})_2\text{D}_3$ is seen [125]. In proximal tubule human kidney epithelial cell line, HK-2G, the CaSR also mediates expression of the vitamin D receptor (VDR) via phosphorylation of the MAPK, p38, which regulates the VDR promoter, another way by which it may regulate the effects of $1,25(\text{OH})_2\text{D}_3$ [126]. Inhibiting p38 phosphorylation or suppressing CaSR expression, both impeded VDR induction. Interestingly, Vitamin D is also able to regulate expression of CaSR in this region [127].

In the thick ascending limb (TAL) of Henle’s loop, the CaSR is expressed on the basolateral membrane and regulates divalent cation urinary excretion, by regulating the activity of channels, transporters and exchangers involved in ion transport during hypercalcemia [122].

The CaSR is expressed in the distal convoluted tubule where it responds to changes in urinary $\text{Ca}^{2+}$ [122]. High urinary $\text{Ca}^{2+}$ activates the CaSR initiating PKC signalling which allows coupling to the TRPV5 channel. TRPV5 allows $\text{Ca}^{2+}$ into the lumen, preventing excessive $\text{Ca}^{2+}$ loss in the urine [128].
Juxtaglomerular cells also express the CaSR where it regulates the release of the enzyme renin, which is involved in blood pressure regulation [122, 129]

1.9.2 Bone

The effects of CaSR-mediated regulation of PTH secretion on bone metabolism have been well documented [130]. Bone metabolism involves osteoblasts mediating the formation of bone, and bone resorption by osteoclasts. Mature osteoclasts express the CaSR and their activity is responsive to changes in $\text{Ca}^{2+}$ and other CaSR agonists within their microenvironment [131]. The osteoclast precursor murine cell line RAW 264.7, was found to express the CaSR which allowed migration of these cells to the bone in a $\text{Ca}^{2+}$-dependent manner via PI3K/Akt and PLCβ pathways [132]. The migration of these cells to bone is thought to be one of the initial steps for bone remodelling to commence.

Although CaSR expression in osteoblasts has been contentious, CaSR expression has been confirmed in the rat osteoblast cell line MC3T3-E1 cell line [133]. CaSR activation by $\text{Ca}^{2+}$ or other cationic agonists stimulates phosphorylation of MAPK, ERK1/2, p38 and JNK in this cell line, promoting mitogenesis [134]. The CaSR also promotes chemotaxis of the same cell line via $G_q$ and $G_i$ signalling, which promote PLC activation [135].

Because of the effects of CaSR on bone remodelling it is a therapeutic target for the treatment of bone metabolism disorders. Indeed the drug, strontium renelate, used as a treatment for post-menopause osteoporosis, mediates some of its bone proliferative effects via CaSR-mediated ERK1/2 phosphorylation [136, 137]. $\text{Sr}^{2+}$ was found to induce proliferation in rat primary osteoblast cell line, which was CaSR-mediated as introducing a dominant negative CaSR mutant attenuated the proliferative response [136]. However, calcimimetics and
calcilytics are limited in applications of bone remodelling due to their effect on non-skeletal tissues [130].

1.9.3 Breast

The effects of Ca\(^{2+}_o\) on proliferation of breast epithelia prompted the investigation of CaSR expression in breast tissue [138]. CaSR mRNA transcript and protein expression was found in normal and abnormal breast epithelial cells.

The CaSR appears to play an important role in regulating PTHrP secretion in breast tissue. As mentioned above, switching of CaSR G-protein coupling leading to enhanced PTHrP secretion may contribute to breast cancer [117]. In other breast cancer lines, CaSR-mediated PTHrP secretion had osteolytic effects on bone resulting in increased Ca\(^{2+}_o\) further stimulating CaSR activity and subsequent osteolysis, a detrimental cycle potentially responsible for osteolytic metastases [139]. The metastases arises from the migration of breast cancer cells to the bone, which is a result of increased Ca\(^{2+}\) levels around the bone environment acting as a chemoattractant [140]. The CaSR’s activation of ERK1/2, MAPK and PLC\(\beta\) signalling in response to elevated Ca\(^{2+}_o\) is involved in the migratory response.

There is an increase in CaSR expression on mammary epithelia which is involved in the regulation of PTHrP secretion by the CaSR and also regualtes the Ca\(^{2+}\) transport to milk during lactation according to Ca\(^{2+}\) availability in the body [141, 142].

1.9.4 Prostate

PTHrP secretion from prostate cancer cells in response to elevated Ca\(^{2+}_o\) is CaSR-mediated, and like in breast cancer cells, promotes osteolysis [143]. In the PC-3 prostate cancer cell,
activation of CaSR, promoted ERK1/2 phosphorylation via EGRF activation, which resulted in PTHrP secretion [144].

1.9.5 Stomach

CaSR expression in the stomach is largely in the gastric glands where it is involved in regulating acid and hormone secretion and the repair of mucosal epithelia [145]. It is largely expressed on the basolateral membrane [146]. In human gastric glands, cationic activation of the CaSR induces activity of the acid secreting H⁺-K⁺-ATPase, possibly via a PI-PLC mediated pathway [147]. CaSR activation of PI-PLC in G-cells promotes influx of Ca²⁺, stimulating gastrin secretion [148, 149]. CaSR activity also promoted cell proliferation of gastric mucosal epithelial cells, implying a role of the CaSR in acid damaged epithelia [146].

1.9.6 Colon

In colon carcinoma cell lines, CaSR has a chemopreventative role, by regulating differentiation and proliferation [150, 151]. In a comparison of normal colon and carcinoma cell lines, there was a reduction in CaSR expression in differentiated tumour cell lines compared to normal cells. There was no CaSR expression in undifferentiated tumours. Furthermore, CaSR activation promoted E-cadherin expression and suppressed β-catenin/T-cell factor, effects which are thought to prevent cancer [151]. In Caco-2 colon carcinoma cell lines, the CaSR activation was able to prevent release of the cell from G1/S phase control, which occurs at low Ca²⁺₀, thereby preventing carcinoma proliferation [152]. In the CBS colon carcinoma cell line, the CaSR’s chemoprotective role was due to increased ERK1/2 phosphorylation in response to CaSR activation and upregulated CaSR expression [153].
1.9.7 Heart and blood vessels

In the cardiovascular system, CaSR expression is found in tissues of the heart and blood vessels [154]. In rat cardiomyocytes, the CaSR mediates Ca\(^{2+}\r_o\)-induced Ca\(^{2+}\r_i\) mobilisation via PLC. The CaSR also mediates ERK1/2 phosphorylation in these cells via G\(_{\alpha 12}\) [155] and modulates cell proliferation [154].

In blood vessels, CaSR activation by Ca\(^{2+}\r_o\), Mg\(^{2+}\r_o\) or neomycin had a bi-phasic effect, inducing first constriction and then relaxation implicating the CaSR in blood pressure control [154].

1.9.8 Other tissue

CaSR is expressed in both neuronal and glial cells in various organs and nuclei of the CNS and its levels change during the lifecycle [156]. In addition to its roles in development, the CaSR contributes to control of salt and water metabolism modulates synaptic signalling and cell growth.

The CaSR is also expressed in taste tissues in various mammals and contributes to kekumi taste in humans [157].

1.10 CaSR signalling

Although various CaSR-mediated signalling pathways have been identified together with their physiological significance, very little is known about the receptor-based mechanisms that support the control of ligand-dependent signalling pathways. These events include interactions between subunits across the dimer interface, together with selection of G-proteins for activation.
Studies across the GPCR superfamily have highlighted the roles of intracellular loops (iLs) and C-tail in G-protein selection and coupling [101]. Consistent with this, CaSR-mediated PI-PLC signalling is dependent on residues in iL-2, iL-3 and the C-tail, but the role of these residues in other signalling pathways has not been investigated [42, 46]. It has also been established that interactions between subunits across the dimer interface are required for the normal control of signalling [78]. However, the specific subunit requirement for the normal sensing of Ca$^{2+}$, or other ligands or signalling via different pathways are unknown. I set out in the current study to investigate the impact of mutating a single subunit in CaSR dimers with a view to finding answers to these basic questions.
1.11 Aims

**Aim 1: Are CaSR residues and domains critical for PI-PLC signalling, important for other CaSR signalling pathways?**

To determine the roles of iL residues: F706 (iL-2), L797 (iL-3), E803 (iL-3) and the C-terminal in CaSR-mediated signalling using HEK-293 cells transiently transfected with alanine mutants or the truncation mutant 866X, on IP1 accumulation, Ca$^{2+}$i mobilisation, ERK1/2 phosphorylation and suppression of cAMP levels.

The work undertaken with respect to this aim is reported in Chapter 3.

**Aim 2: Can the formation of CaSR heterodimers at the cell surface be regulated?**

To develop a heterodimer expression system composed of a CaSR-GABA$_{B1}$ chimeric receptor that would be retained intracellularly when expressed alone, and a CaSR-GABA$_{B2}$ chimeric receptor that would traffic CaSR-GABA$_{B1}$ to the cell surface to permit the analysis of mutant CaSR heterodimers.

The work undertaken with respect to this aim is reported in Chapter 4.

**Aim 3: What are the minimum subunit requirements for CaSR signalling?**

Utilising the CaSR-GABA$_{B}$ heterodimer expression system developed in Aim 2, I studied the behaviour of mutant heterodimers possessing inactivating mutations in the VFTD (G143E or R185Q) or the HHD (F706A, E803A or L797A) using Ca$^{2+}$o-induced Ca$^{2+}$i mobilisation to determine the minimum subunit requirements for Ca$^{2+}$o sensing and signalling.

The work undertaken with respect to this aim is reported in Chapter 5.
Experimental Procedures
Chapter 2  Experimental Procedures

2.1  Molecular Biology

2.1.1  Plasmids

The wild-type CaSR cDNA (cassette version) [86], which had previously been cloned into the Kpn I and Xba I sites of pcDNA3.1(+) (Invitrogen), 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).

The GABA_{B1} and GABA_{B2} plasmids were a kind gift from Dr. David Hampson (Graduate Department of Pharmaceutical Sciences, University of Toronto, Canada).

Mr. Roy Chan generated the CaSR_{908}-B1, CaSR-B2 and the R185Q-B2 plasmids.

The FLAG-tagged CaSR plasmid was generated by Miss Sue Ling. The FLAG epitope, DYKDDDDK, was introduced between residues 371 and 372 of the WT CaSR plasmid.

Mr. James Ward introduced the F706A, L797A and E803A mutations into the CaSR and the CaSR-FLAG plasmids by site-directed mutagenesis.

The plasmid encoding a FRET based EPAC construct to monitor cAMP levels was a kind gift from Dr. Kees Jalink (Division of Cell Biology, The Netherlands Cancer Institute) [158].

The pcDNA3.1(+) mammalian expression vector was purchased from Invitrogen (Green Island, New York, USA).

A number of strategies were employed to generate CaSR mutants and chimeras which are summarised in (Figure 2.1).
Figure 2.1 Summary of cloning strategies employed to generate CaSR constructs
Site-directed mutagenesis PCR was used to introduce point mutations. To generate new restriction sites or truncations, PCR amplification of parental DNA was performed with the PCR product possessing the required restriction sites and/or truncations. Digested PCR product was ligated into the vector backbone. Chimeric constructs were generated by digesting the relevant DNA segment with restriction enzymes and ligating it into a new vector backbone.
2.1.2 Bacterial culture media

Bacterial cells were cultured in Luria Bertani (LB) media or LB-agar plates. LB broth was prepared by adding five LB media tablets (MP Biomedical; Seven Hills, NSW, Australia) to 200 mL of milliQ H₂O (Millipore; Australia) and 1 mM NaOH followed by autoclaving. For antibiotic selection of colonies, 100 μg/mL of ampicillin (Sigma Aldrich, Australia) was added after autoclaving.

LB agar plates were prepared in a similar manner to LB broth, with the addition of 1.5% (w/v) of bacteriological grade agar powder (Mo Bio Laboratories; California, USA) prior to autoclaving. After autoclaving, antibiotics were added as required and the warm media was poured into 90 mm petri dishes to a depth of 5 mm.

2.1.3 Preparing chemically competent DH5α E. coli cells

DH5α Escherichia coli cells obtained from Dr. Alison Franks, had previously been prepared as a glycerol stock that was stored at -80°C. The glycerol stock was streaked out on an antibiotic free LB agar plate and incubated overnight at 37°C. A single colony was inoculated in 5-10 mL of antibiotic free LB broth and cultured overnight at 37°C, shaking at 200 rpm. The next day the culture was diluted in 200 mL of fresh LB broth such that the starting OD₆₀₀ was ~0.01. The culture was grown until OD₆₀₀= 0.6-0.7 at which point it was chilled in an ice-slurry for 1 h. The cells were pelleted by centrifuging the culture at 5000 g for 5 min at 4°C, and resuspended in 50 mL of sterile, ice-cold MgCl₂ by gentle shaking in an ice-bath for 30 min. The cells were pelleted again at the same conditions and resuspended in 100 mL of sterile, cold 100 mM CaCl₂ by gentle shaking in an ice-bath for 90 min. The cells were pelleted a final time, resuspended in 10 mL of sterile, cold 85 mM CaCl₂ with 15% (v/v) glycerol. The suspension was dispensed and stored as 200 μL aliquots at -80°C.
2.1.4 Transformations

DH5α cells were transformed with DNA using the heat pulse method. 2-5 μL of DNA stock, ligation reaction or Dpn I treated site-directed mutagenesis PCR reaction was added to the bottom of a pre-chilled 15 mL falcon tube and incubated with 50 μL of thawed, chemically competent DH5α cells for 30 min on ice. The transformation reaction was then heat shocked by placing the tube in a 42°C water bath for 2 min before briefly returning to ice. The reaction was cultured in 200 μL of antibiotic free LB broth at 37°C for 40 min in a shaker at 200-250 rpm. 50-250 μL of the transformation reaction was then plated out on LB agar plates (100 μg/mL ampicillin) and incubated overnight at 37°C.

2.1.5 Plasmid DNA Purification

Plasmid DNA was purified either as minipreps or midipreps using the QIAprep Spin Miniprep kit and HiSpeed Plasmid Midiprep kit from Qiagen (Australia), respectively. Briefly, transformed bacterial cells were inoculated in 4-6 mL of LB broth (100 μg/mL ampicillin) for minipreps or 50 mL of broth for midipreps, and cultured overnight at 37°C, shaking at 200 rpm. The cells were pelleted by centrifugation at 6000 rpm for 1 min for minipreps and 15 min for midipreps. Alkaline lysis of the pelleted cells followed with membrane and proteins precipitated out and removed by centrifuging or filtering. DNA was then precipitated with isopropanol and washed with ethanol before being dissolved in EB (10 mM Tris-Cl, pH8.5) buffer for minipreps or TE Buffer (10 mM Tris-Cl, 1 mM EDTA, pH8.0) for midipreps. Mini spin columns with lids from Epoch Biolabs (Texas, USA) were used for miniprep purifications and Buffer N3 was often replaced with Buffer P3 from the midiprep kit.
2.1.6 DNA Ligations

Ligations were performed using T4 DNA Ligase (New England Biolabs; Massachusetts, USA). DNA fragments purified from PCR amplification or restriction digestion were ligated in a 3:1-5:1 concentration ratio of insert to vector. The ligation reaction consisted of 20 units of T4 DNA ligase in the presence of 1x supplied buffer [50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM dithiothreitol, 1mM ATP]. The reaction was made up to 10 μL with milli-Q water and incubated at 16°C for 30-120 min.

2.1.7 Restriction Enzyme Digestion

All restriction enzyme digestions were performed using restriction enzymes supplied from New England Biolabs (MA, USA). Briefly, DNA from minipreps, midipreps or PCR amplification reactions was digested with 10-50 U of restriction enzyme (Kpn I, Xba I, Bam HI, and Xho I) in the optimal buffer conditions at 37°C for 1-16 h. Reactions were set up in final volumes of 10 or 50 μL.

2.1.8 Primer Design

Primers were designed for PCR amplification to bind 20-30 bp of template DNA, with a GC content of 40-60% and a T_m greater than 78°C. When required the primers would contain additional 5' or 3' sequences of restriction sites, to facilitate cloning into vectors, or antibody epitopes.

Primers for site-directed mutagenesis were designed according to the QuickChangeII site directed mutagenesis kit (Agilent technologies; California, USA). Briefly, the primers were designed to be ~25-45 bp in length with the desired mutations located in the middle of the primers. The GC content was greater than 40% and the T_m was greater than 78°C.
2.1.9 **PCR amplification**

PCR amplification was performed using *PfuUltra* II Fusion HS DNA polymerase (Agilent technologies; California, USA) or MyTaq HS DNA Polymerase (Bioline; Redfern, Australia). Reactions performed with *PfuUltra* II Fusion HS DNA polymerase were set up containing 0.2-2 ng/μL of template DNA, 0.2 μM of forward and reverse primer, 250 μM of each dNTP, 1x reaction buffer, 1.25 U of the polymerase and were made up to 50 μL with milliQ H₂O. The PCR reactions were held at 95°C for 2 minutes, then cycled with a denaturing temperature of 95°C for 20 sec, annealing temperature of primer Tₘ-5°C for 20 sec and an extension temperature of 72°C for 15 sec/kb; 30 cycles were performed. The block was then held at 72°C for 3 min. Once the PCR was complete, reactions were kept at 25°C or stored at 4°C until required.

Reactions with MyTaq HS DNA Polymerase contained 50 pg-10 ng of template DNA, 20 μM each of the forward and reverse primers, 1x supplied buffer (containing 5 mM dNTPs, 15 mM MgCl₂), 5 U MyTaq HS DNA Polymerase and were made up to 50 μl with MilliQ water. The reactions were initially held at 95°C for 1 min, and then cycled 35 times with denaturing at 95°C for 15 sec, annealing at 53-65°C for 15 sec and extension at 72°C for 10 s/kb. The block was then held at 72°C for 3 min. Once the PCR was complete, reactions were kept at 25°C or stored at 4°C.

2.1.10 **Site-directed mutagenesis**

Site-directed mutagenesis was performed using the Quikchange II site-directed mutagenesis protocol and *PfuUltra* II Fusion HS DNA polymerase (Agilent technologies; California, USA). Site-directed mutagenesis reactions were set up in a final volume of 25 μL with 5-50 ng of template DNA, 0.2 μM each of forward and reverse primer, 250 μM dNTP, 1.25 U of
*PfuUltra* II Fusion HS DNA polymerase in 1 x supplied buffer. The PCR was performed in Eppendorf thermocycler with the block initially held at 95°C for 30 sec, then 18 cycles were performed with denaturing occurring at 95°C for 30 sec, primer annealing occurring at 50-65°C for 1 min and extension at 68°C for 1 min/kb of plasmid. The block was then held at 68°C for 10 min and then the reactions were kept at 25°C or 4°C until required.

### 2.1.11 Agarose Gel Electrophoresis and Gel Extraction

1% (w/v) agarose gels were prepared by dissolving agarose powder (Promega, USA) in 1x TAE buffer (0.04 M Tris, 0.02 M acetic acid, 1 mM EDTA, pH 8.0) by heating in a 800W microwave (Samsung, Australia). A 50 mL gel solution was heated for 1.5 min with an additional heating of 30 sec/ 50 mL of solution. The solution was cooled before 0.04 μg/mL ethidium bromide (EtBr; Gibco, USA) was added. The gels were poured to a thickness of ~10 mm and set with combs of either 3 mm or 8 mm wide teeth to a depth of 8 mm. Once set, the gel was covered with TAE buffer (0.04 μg/mL EtBr). Five volumes of DNA sample with one volume of 6x loading dye (New England Biolabs, USA) were loaded into each well. The 1kb DNA ladder (New England Biolabs, Massachusetts, USA; Figure 2. 2) was loaded to estimate fragment sizes. Electrophoresis of samples was performed at 9 V/cm for 60-80 min. Electrophoresis of DNA to be extracted from the gel was performed at 8 V/cm for 60-120 min. The BioRad UV Transilluminator (BioRad, Australia) was used to visualise DNA bands under UV light, and gel images were documented using the Quantity One software (BioRad, Australia). DNA was extracted from gels using the QIAquick gel extraction kit (Qiagen, USA).
The DNA ladder was electrophoresed with samples on agarose gels and used to estimate plasmid DNA band sizes. Digestion of the WTCaSR plasmid with Kpn I and Xba I yielded bands of 5.4 kb and 3.2 kb in size corresponding to the pcDNA3.1 vector and the CaSR cDNA insert, respectively.

### 2.1.12 DNA Quantification

DNA quantification for quality and concentration was performed either on the Ultrospec 1000E spectrophotometer (Pharmacia Biotech, USA) or the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA).

DNA samples were prepared as 1/30 dilution in a final volume of 150 μL in MilliQ water for measurement in the Ultrospec 1000E. Absorbances at 260 (A$_{260}$) and 280 nm (A$_{280}$) were measured using a quartz cuvette (Starna Pty. Ltd., Thornleigh, NSW, Australia) with a 1 cm light path. DNA concentration was calculated using the following equation:

\[
[DNA] \text{ (ng/μL)} = A_{260} \times 50 \text{ ng/μL} \times 30 \text{ (dilution factor)}
\]
DNA quality was assessed using the $A_{260}:A_{280}$ ratio with a value between 1.8-2.0 indicative of DNA with sufficient purity.

Alternatively, 1-2 μL of stock DNA solutions was placed directly on to the Nanodrop 1000 spectrophotometer. Concentration and quality quantification was computed by the Nanodrop 1000 Version 3.6.0 software.

2.1.13 DNA sequencing

For DNA sequencing of plasmids, 600-1500 ng of DNA sample was submitted with 9.6 pmol of sequencing primer made up to 12 μL with MilliQ water to the Australian Genome Research Facility (AGRF; Brisbane or Sydney node, Australia) where sequencing was performed. Primers were designed to bind at least 100 bp upstream of the region of interest with a length of 20-30 bp, GC content of ~40-60% and a $T_m$ of ~55°C. Briefly, Sanger sequencing was performed by PCR of the sample with the primer where the block was held at 96°C for 2 min, before performing 30 cycles where denaturing occurred at 96°C for 10 sec, primer annealing occurred at 50°C for 5 sec and extension was performed at 60°C for 4 min. The block was then held at 4°C.

Sequencing results were retrieved from the AGRF website and alignment was performed using the SerialCloner 1.3r11 software (SerialBasics).

2.2 Cell Culture

2.2.1 HEK-293 Cells

HEK-293 cells were used in this study (ATCC; Virginia, USA). The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Australia) supplemented with 10%
FBS, 25 Units/ ml Penicillin and 25 μg/ml of Streptomycin (Gibco, Australia). The cells were maintained in 25 cm$^2$ or 75 cm$^2$ tissue culture flasks (Corning Life Sciences; Massachusetts, USA) at 37°C, in 5% CO$_2$ humidified incubator.

The cells were subcultured upon reaching 90-100% confluency. Cells were incubated with 0.25% Trypsin-EDTA (Gibco, Australia) for 5 min at room temperature for detachment from the surface of the flask. To minimise cell damage, the detached cells were transferred to 20 mL fresh DMEM to neutralise the trypsin. The cells were then pelleted by centrifugation at 1500 rpm for 3 min. The supernatant was decanted and the pellet resuspended in fresh medium. The cell suspension was then transferred to sterile plates, culture flasks or coverslips, as required.

### 2.2.2 Transient Transfection

HEK-293 cells were transiently transfected using the X-tremeGENE HP DNA Transfection Reagent (Roche, Germany) at room temperature; under sterile conditions 48-72 h prior to experimentation (Figure 2.3). A total of 0.5-1 μg of DNA was diluted in 100 μL of DMEM and gently vortexed, then 3 μL of transfection reagent was added to the diluent and gently mixed and incubated for 15 min to allow the transfection reagent and DNA to form complexes. This transfection solution was added to different culture vessels as required (Table 2.1).
Figure 2. 3 Protocol for transfecting HEK-293 cells with XtremeGENE HP
Briefly, DNA was added to serum-free DMEM and incubated with XtremeGENE HP for 15 min. The DNA-XtremeGENE containing transfection solution was then added to subcultured HEK-293 cells as required. Transiently transfected HEK-293 cells were used for subsequent experiments, typically 48 h after transfection.
Table 2.1 Amount of transfection solution added to HEK-293 cells grown in different culture vessels

For transient transfections of HEK-293 cells, XtremeGENE HP was incubated plasmid DNA in a 6 μL/100ng ratio and added to subcultured cells as required.

<table>
<thead>
<tr>
<th>1-well of culture vessel</th>
<th>Surface Area (cm²)</th>
<th>Total volume of media (mL)</th>
<th>Volume of transfection solution to add (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well plate</td>
<td>0.3</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>24 well plate</td>
<td>1.9</td>
<td>0.5</td>
<td>50</td>
</tr>
<tr>
<td>6 well plate</td>
<td>9.4</td>
<td>2</td>
<td>200</td>
</tr>
</tbody>
</table>

2.3 Expression ELISA

2.3.1 Poly-D-Lysine Coating of 96-well plates

Each well of a sterile 96-well plate was coated with 50 μL of a 25 ng/mL poly-D-lysine solution (Sigma Aldrich, Australia) and incubated overnight at 37°C in 5 % CO₂ incubator. Excess solution was decanted from the plate and each well was washed with 100 μL sterile MilliQ water. The plate was then air dried under sterile conditions and stored at 4°C.

2.3.2 4% (w/v) Paraformaldehyde Solution

Paraformaldehyde (PFA) solution was prepared by dissolving 4 g of PFA powder (Sigma Aldrich, Australia) in 50 mL MilliQ water with 1 mL of 1M NaOH at 60°C by stirring. 10 mL of 10x phosphate-buffered saline (1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄, pH 7.4) was added and the solution was allowed to cool before adjusting the pH to 7.4. The solution was made up to 100 mL and stored as 6 mL aliquots at -20°C. Prior to use, aliquots were thawed out.
2.3.3 **Culturing cells on to Poly-D-Lysine coated plates**

HEK-293 cells grown to 90% confluency in 6-well plates or 96-well plates were transiently transfected 48 h prior to assaying. In the case of 6-well plates, each well was then subcultured in to 16 wells of a poly-D-lysine coated plate 24 h prior to assaying by detachment with 200 μL of 25% trypsin-EDTA. Detached cells were then suspended in 10 mL of fresh growth media, and the cells pelleted by centrifugation at 1500 rpm for 3 min. The supernatant was decanted and the pellet resuspended in 3.2 ml of fresh growth media. 200 μL of the cell suspension was transferred to each well of the 96-well plate.

2.3.4 **Expression ELISA**

Working on ice, media was aspirated from each well of a 96 well plate of transiently transfected HEK-293 cells, and washed with 200 μl of cold TBS-T buffer (0.05M Tris, 0.15M NaCl, 0.05 % (v/v) Tween-20, pH 7.4). The cells were fixed by incubating with 100 μL of 100% methanol, or 4% PFA solution for 15 min. The fixatives were then removed and the cells washed again with 200 μl TBS-T buffer. All subsequent steps were carried out at room temperature. Each well was incubated with 100 μL of 1% (w/v) skim milk blocking solution (Diploma-Fonterra Foods, North Ryde, Australia) for 1 h with gentle rocking, followed by incubation with 100 μl of 1:5,000 dilution of the monoclonal anti-FLAG M2 horse-radish peroxidase (HRP)-conjugated antibody (Sigma Aldrich, Australia) in TBS-T for 1 h. The cells were then washed three times with TBS-T and incubated with 100 μL tetramethylbenzidine (TMB; Sigma Aldrich, Australia) liquid substrate in the dark for 12 min. The substrate reaction was stopped by the addition of 100 μL 1 M HCl. 150 μL of the reaction supernatant was transferred to a new plate and absorbance at 450 nm was measured by the Wallac EnVision 2103 multilabel reader (Perkin Elmer, USA) using the Wallac EnVision Manager software (version 1.08).
2.4 IP-one Assay

Intracellular IP₁ was assayed using a homogeneous time-resolved FRET (HTRF) kit (Cisbio Bioassays, France). HEK-293 cells were subcultured and transfected in 6-well plates. 24 hours after transfection, the media was removed and the cells detached with 200 µl of 0.25% trypsin-EDTA, and neutralised in 5-10 mL DMEM (10% FBS; 1/200 Penicillin, streptomycin). The cells were centrifuged at 1500 rpm for 3 min. The supernatant was removed and the pellet re-suspended in 1.2 mL of DMEM (10% FBS; 1/200 Penicillin, streptomycin). 30 µl of cell suspension was added to each well of a sterile 384 well Optiplate (Perkin Elmer, USA) and the cells cultured for a further 24 h. The media was then removed and the cells incubated with 14 µL of agonist in stimulation buffer (10 mM Hepes, 0.5 mM MgCl₂, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl, pH 7.4) for 30 min followed by 3 µL/well of a 1 in 20 dilution of anti-IP₁ cryptate Tb conjugated antibody in 1 x lysis buffer and 3 µl/well of a similarly prepared solution of IP₁-d2 conjugate. Antibody incubation was performed overnight and the plate read on the EnVision plate reader with the HTRF protocol. The F₆₁₅/F₆₆₅ ratio was computed and the results analysed on GraphPad Prism.

2.5 Fluorescence Microscopy

Fluorescence microscopy of transiently transfected HEK-293 cells, cultured on coverslips in 24-well plates, was used to evaluate their intracellular calcium mobilisation and cAMP suppression responses to increasing [Ca²⁺ᵦ].


2.5.1 Fura-2 AM loading

Fura-2 AM (Sigma Aldrich, Australia) is a fluorescent probe used to monitor cytosolic Ca\(^{2+}\). Fura-2 AM itself is not fluorescent, however it is able to penetrate through the cell membrane into the cytosol where it is cleaved by intracellular esterases to yield Fura-2, which is fluorescent.

HEK-293 cells were loaded with 5 µM Fura-2 AM in loading solution [physiological saline (PSS; 125 nM NaCl, 4 mM KCl, 20 mM HEPES, 0.1% D-Glucose, 1 mM MgCl\(_2\)), with 1 mM CaCl\(_2\), 0.8 mM NaH\(_2\)PO\(_4\), and 0.1% (w/v) bovine serum albumin (BSA)]. After incubation for 90-120 min at 37ºC in the dark, the Fura-2 solution was removed. The cells were then washed and stored in loading solution.

2.5.2 Ca\(^{2+}\) Epifluorescence microscopy

Fura-2 loaded HEK-293 cells on cover slips were loaded in a closed bathing chamber (70 µl volume; Warner, USA) and mounted on the stage of the Zeiss Axiovert 200M inverted, fluorescence microscope (Zeiss, Germany). Solutions containing CaSR ligands were perfused through the chamber at a constant rate of 1-3 ml/min using the VC-8 perfusion Channel Control System (Warner, USA) and the Gilson Minipuls 2 pump (Figure 2. 4). Cell samples were observed using the plan-Neoflar 63x/0.95 Korr. or 40x/ 0.6 Korr objective lens (Zeiss, Germany). The samples on stage were excited alternately by the Lambda DG-4 light source (Sutter Instruments, USA) at 360 nm and 340 nm, and the emission at 510 nm was captured by the Axiocam HSm (Zeiss, Germany) every 1000 ms and computed with the Slidebook software (Intelligent Imaging Solutions; Colorado, USA).
2.5.3 cAMP Microscopy

HEK-293 cells were plated out on to sterile coverslips in 24 well plates. 48 hours prior to microscopy, cells were transfected with WT CaSR or a mutated CaSR plasmid and co-transfected with a cAMP fluorescence resonance energy transfer reporter construct: CFP-EPAC-YFP-YFP.

The media was replaced with 0.5 mM Ca$^{2+}$ in PSS 15 mins prior to microscopy. The cells were placed in the closed bathing chamber and loaded on to the microscope stage as described in section 2.5.2. The fluorescence response was monitored upon perfusion of solutions through the chamber. Cells were excited at 436 nm for 500 ms and emission at 488 nm and 528 nm corresponding to F$_{CFP}$ and F$_{YFP}$, respectively collected every 1000 ms.
Figure 2.4 Perfusion setup for fluorescence microscopy.

Perfusion solutions were kept warm in the temperature control cabinet at all times during microscopy. Inlet tubing connected to an inlet pump allowed for solutions to carry from the temperature control cabinet through to the microscope stage. On the microscope stage, a stage adaptor held live cells on coverslips in a cell chamber (shown inset). Solutions were fused through the cell chamber and out through the outlet tubing connected to the outlet pump after which the solution waste was collected. The microscope was connected to the DG-4 Lambda light source which contained a Xenon lamp and excitation filters for fluorescence.

2.5.4 Perfusion Solutions

Perfusion Ca\(^{2+}\) solutions for microscopy were made up in PSS by diluting 1 M CaCl\(_2\) stock to appropriate concentrations. Forskolin (Sigma Aldrich, Australia) was dissolved in DMSO to a 50 mM stock, which was diluted in PSS as required.
2.6 ERK Alphascreen assay

To measure the level of ERK1/2 phosphorylation an alphascreen SureFire assay kit was used (Perkin Elmer, USA). HEK-293 cells were subcultured and transfected in poly-D-lysine coated 96 well plates. 48 hours after transfection the media was replaced with DMEM (0.2% BSA, 1.5 mM CaCl₂) to serum starve the cells for 4-14 hours. Cells were then washed once with PSS containing 0.2 mM CaCl₂ and then incubated with 90 µL/well of 0.2 mM CaCl₂ containing PSS at 37°C to Ca²⁺ starve the cells. After 30 min, 10 µL of 10x agonist solutions were added and the cells incubated for a further 10 min. The solutions were then removed and 50 µL/well of 1 x supplied lysis buffer was added and the plate agitated at 150 rpm for 10-15 min on a shaker. 5 µL of cell lysate was transferred to a white, 384 well Optiplate (Perkin Elmer, USA) and incubated overnight with acceptor and donor beads diluted as specified in the protocol. The plate was read with the alphascreen protocol on the EnVision plate reader.

2.7 Data Analysis and curve fitting

All data are presented as mean ± standard error of the mean (SEM), unless otherwise stated.

2.7.1 Expression Elisa

The experiment was performed in quadruplicates for each condition. After the averaged background (vector-only transfected HEK-293 cells) was subtracted, the results were normalised to total WT expression as 100%. The mean of the replicates and SEM were calculated and data from independent days were grouped. The group mean and SEM were calculated and in GraphPad Prism.
2.7.2 \( \text{Ca}^{2+}_{\text{i}} \) mobilisation

Raw \( F_{340}/F_{380} \) ratio data was collected by the Stallion software for 10 regions of interest (ROI) per experiment. The ROI had their raw data integrated to determine the area under the curve corresponding to the response for each individual \( \text{Ca}^{2+}_{\text{o}} \) concentration. The control background (0.5 mM \( \text{Ca}^{2+}_{\text{o}} \)) was subtracted from the responses to give integrated fluorescence response units (IFRUs). The integrated responses were averaged for each experiment. The average integrated response from independent days was grouped and the mean of the grouped data and SEM were calculated in Excel. The mean response was fitted in GraphPad Prism software to a modified form of the Hill equation:

\[
F(x) = d + \frac{(a - d) \times x^b}{c^b + x^b}
\]

where \( a \) = maximal response (\( E_{\text{max}} \)); \( b \) = Hill co-efficient; \( c \) = half maximal effective concentration (\( \text{EC}_{50} \)); \( d \) = minimum response.

All four parameters in addition to the Log\( \text{EC}_{50} \) were calculated according to best fit in GraphPad prism, with standard errors.

2.7.3 cAMP microscopy

\( F_{\text{CFP}}/F_{\text{YFP}} \) was computed by the Stallion software for 10 ROIs. The fluorescence ratio of the last minute of exposure to each solution was averaged and after background (0.5 mM \( \text{Ca}^{2+}_{\text{o}} \) control) subtraction was expressed as a percentage of the Foskolin-stimulated ratio (100%). The data from independent days was grouped and the mean and SEM calculated in Excel. The concentration–response curves were fitted in GraphPad Prism according to the following modified form of the Hill equation:
Y = Bottom + (Top - Bottom) / (1 + 10^((LogIC_{50} - X) * HillSlope))

Estimates of the curve fit parameters and their standard errors were calculated in GraphPad Prism.

### 2.7.4 IP₁ assay

Each experiment was performed in triplicates. The F₆₁₅/F₆₆₅ ratio was calculated and data from independent days were grouped and expressed as fold-change with respect to WTCaSR control (0.5 mM Ca²⁺₀) after 0.5 mM Ca²⁺₀ background subtraction. The mean and SEM of the grouped data were calculated in GraphPad Prism to generate a concentration-response curve, to which the modified Hill equation was fit (refer to 2.7.1).

All curve-fit parameters in addition to the LogEC_{50} were calculated according to best fit in GraphPad prism, with standard errors.

### 2.7.5 ERK1/2 phosphorylation

Each experiment was performed in triplicates. The fluorescence counts were expressed as fold-change with respect to WTCaSR control (0.5 mM Ca²⁺₀) after 0.5 mM Ca²⁺₀ background subtraction. The mean data from independent days was grouped and the grouped mean and SEM were calculated in GraphPad Prism to generate a concentration-response curve, to which the modified Hill equation was fit (refer to 2.7.1).

All curve-fit parameters in addition to the LogEC_{50} were calculated according to best fit in GraphPad prism, with standard errors.
2.8 Statistical Analysis

Statistical analysis of data was performed using analysis of variance (ANOVA), unless stated otherwise, in GraphPad prism with the statistical significance threshold $p<0.05$ to assign significance. The Log of $EC_{50}$ or $IC_{50}$ values and the standard error of the Log-value was used for statistical analysis of differences in $Ca^{2+}_o$-sensitivities.
Chapter 3
Chapter 3  The molecular basis of G-protein coupling

3.1 Introduction

GPCR signalling mediated by heterotrimeric G-proteins requires direct and indirect interactions between the membrane-embedded receptor and cytoplasmic G-proteins [159]. To be able to signal via G-proteins, the receptor requires determinants that enable it to couple to and activate G-proteins, as well as determinants of G-protein selectivity [160].

3.1.1 GPCR requirements for G-protein coupling

In response to extracellular changes, GPCRs activate heterotrimeric G-proteins to relay the information to the intracellular environment [101]. Interactions between the GPCR and G-proteins require cytoplasmic regions of the receptor, namely intracellular loop-2 (iL-2), intracellular loop-3 (iL-3) and the C-tail.

In general, studies of GPCRs have shown that interactions between receptors and the $G_a$ require iL-3 and the proximal C-tail, but interactions involving iL-2 are also required for an exchange of GDP to GTP exchange and thus G-protein activation [161]. Indeed, replacing either iL-2 or iL-3 of the glucagon receptor with iL-1 of the dopamine D4 receptor impaired the receptor’s cAMP and Ca$^{2+}$ responses to glucagon by increasing EC$_{50}$ values for glucagon 100-fold, while replacing both of the loops abolished receptor responses [162].

In the receptor for vasoactive intestinal peptide and pituitary adenylate cyclase activating peptide, VPAC1, both, introduction of the point mutation, K322A in iL-3 or E394A in the CTD, reduced adenylyl cyclase (AC) activation by ~50%; the double mutant L332A/E395A reduced the response by ~85% [163].
Chapter 3

The molecular basis of G-protein coupling

Figure 3.1 Schematic representation of G-protein signalling by GPCRs
Activated GPCRS recruit and activate G-proteins resulting in an exchanges of GDP for GTP on the α-subunit and subsequent dissociation of the G-protein heterotrimer. The membrane embedded GPCR, contains three intracellular loops, two of which, iL-2 and iL-3, are important for G-protein coupling and signalling. The C-terminal tail of GPCRs also plays a role in G-protein signalling.

These studies suggest that receptor coupling with G-proteins is complex typically involving individual residues and peptide motifs, in the cytoplasmic domains as well as iL-2 and iL-3 [159, 161]. Direct evidence of the roles of iL-2 and iL-3 in interactions with G-proteins came in the landmark crystal structure of the β2-adrenergic receptor complexed with Gs [164].

3.1.2 Determinants of G-protein coupling specificity in GPCRs
In any given cell there is a large repertoire of G-proteins for GPCR coupling (
Table 3. 1). However, each GPCR couples to a specific sub-set of G-proteins within a particular cellular environment. The molecular basis of this G-protein specificity lies within the same cytoplasmic domains of GPCRs that are required for GPCR signalling, i.e. iL-2, iL-3 and the C-tail [101].

Generation of chimeric receptors, in which specific iLs have been replaced by iLs from donor receptors, have been invaluable for demonstrating the role of iLs in specifying G-protein coupling [101]. For example, replacement of the M₂ muscarinic cholinergic receptor (mAChR) iL-3, which selectively activates Gi, with that of M₁ or M₃, both of which activate Gq, resulted in chimeric receptors that activated Gq [101, 165]. Furthermore, replacement of both iL-2 and iL-3 of either the M₁ or M₂ receptors, with those of the β-adrenergic receptor, which couples to Gs, conferred Gs specificity on the chimera [165].

Within them, the iL-2, iL-3 and CTD regions of receptors contain residues and motifs that are determinants for coupling to different G-proteins. The prototypic class B secretin receptor couples to Gs and Gq, resulting in cAMP elevation and Ca²⁺ mobilisation, respectively [166]. Introducing the double mutations R318A/R321A into iL-3 impaired both responses. Interestingly, however, the K302A/L303A double mutant affecting the same loop selectively impaired the cAMP response but not Ca²⁺ mobilisation.
Table 3.1 Heterotrimeric G-protein families, their members and some of their downstream effectors [167]
(+ refers to stimulation, - refers to suppression).

<table>
<thead>
<tr>
<th>G-protein Family</th>
<th>Subfamily</th>
<th>Common Effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gs</td>
<td>Gs, Golf</td>
<td>+ Adenylyl cyclase + Src tyrosine kinases + Tubulin GTPase + Maxi K channel</td>
</tr>
<tr>
<td>Gi/o</td>
<td>Go1, Go2, Gi2-3, Gz, Gt1/2, Ggust</td>
<td>- Adenylyl cyclase - Ca^{2+} channels + Rap1GAPII-dependent ERK/MAPK + K^+ channels +GTPase of tubulin +Src tyrosine kinases</td>
</tr>
<tr>
<td>Gq/11</td>
<td>Gq, G11, G14, G15, G16</td>
<td>+ PLCβ + p63 Rho-GEF + Bruton's tyrosine kinase + K^+ channels</td>
</tr>
<tr>
<td>G12/13</td>
<td>G12, G13</td>
<td>+ PLD + PLCε + NHE-1 + iNOS + E-cadherin mediated cell adhesion + p115RhoGEF + PDZ-RhoGEF + PKA</td>
</tr>
<tr>
<td>Gβ/γ</td>
<td>β1-5/γ1-12</td>
<td>+ PLCβ - ACI + ACII,IV,VII + PI-3 kinase +JNK +Src kinases +PKD</td>
</tr>
</tbody>
</table>
### 3.1.3 Class C GPCR G-protein coupling and specificity

Studies on Class C GPCRs indicate that their requirements for G-protein coupling and specificity are consistent with findings for GPCR classes A and B [160].

Intracellular loops -2 and -3 play an important role in G-protein signalling by class C GPCRs. HEK-293 cells transiently transfected with mGluR-1α mutants affecting iL-2 and iL-3 exhibited impaired G\(_q\) and G\(_s\) signalling [168]. Furthermore, residues in iL-2 were required for selective coupling. Thus alanine mutants of T695, K697 and S702 impaired PLC activation, whereas P698A or the deletion C694-T695 selectively disabled cAMP accumulation. Similarly, the naturally occurring E775K mutation in iL-3 of mGluR-6, which causes type 1 stationary night blindness, uncoupled the receptor from G\(_o\) signalling while retaining G\(_i\) coupling [169].

The C-tail also plays an important role in class C GPCR-dependent for determination of G-protein specificity [170, 171]. Alternative splice variants of mGluR-1 exhibit different G-protein coupling profiles based on differences in their C-tails [171]. Thus, mGluR-1α with a longer C-tail coupled efficiently to G\(_q\) and G\(_s\), but mGluR-1β with a shorter CTD could not couple to G\(_s\). Furthermore, deletion of the mGluR-1 C-tail selectively abolished G\(_q/11\) but not G\(_i/o\) coupling [55].

### 3.1.4 Determinants of CaSR signalling

In the bovine CaSR, alanine screening identified residues in iL-2 and iL-3 that were important for coupling to PLC [42]. Mutating F707 in iL-2 impaired PLC coupling in response to elevated Ca\(^{2+}\). Although eight of the thirteen residues in iL-3 were found to be important for PLC coupling, alanine mutants of L798, F802 and E804 exhibited the greatest
impairments in the PLC response. Furthermore, truncation after residue 866 in the C-tail abolished PLC signalling even though receptor expression was not affected [45-47].

As mentioned above, the CaSR is a promiscuous GPCR able to couple to a number of G-proteins and signalling pathways. Although the roles of iL-2, iL-3 and CTD residues in CaSR-mediated PLC coupling have been investigated, their roles as molecular determinants of G-protein coupling and selectivity are unknown.

3.2 Results

3.2.1 Generation of Mutants

To assess the roles of residues in iL-2, iL-3 and the C-tail in CaSR-mediated G-protein coupling and selectivity, four mutants were selected based on their reported impact on CaSR-dependent PLC signalling [42, 46] (Figure 3. 2). The three alanine mutants known to impair PLC signalling in bovine CaSR were kindly generated by Mr. James Ward using site-directed mutagenesis of human WTCaSR or CaSR (FLAG) as described in experimental procedures 2.1.10 [42]. One mutation was generated in iL-2, F706A, and two mutations were generated in iL-3, L797A and E803A.

In addition a truncation mutant, CaSR_{866}X mutant, lacking all but three residues of the C-tail was generated by PCR amplification of the WTCaSR or WTCaSR (FLAG) with a forward primer encoding a 5’ Kpn I site and a reverse primer designed to bind up to nucleotide 2595 followed by a stop codon and a 3’ Xba I site, as described in experimental procedures 2.1.9. The PCR product was digested with Kpn I and Xba I and then ligated into the pcDNA3.1(+) vector backbone digested with the same restriction enzymes. The ligation reaction was transformed into chemically competent E. coli DH5α cells and colonies selected ampicillin
resistance. Selected colonies were cultured in LB broth and plasmid DNA purified. Sequencing was used to confirm all mutant constructs.

The mutants were then analysed for their effects on CaSR expression and function.

![Schematic representation of putative signalling mutants in the CaR heptahelical domain.](image)

Figure 3.2 Schematic representation of putative signalling mutants in the CaR heptahelical domain.

Three alanine mutations (black) were introduced in iL-2 and iL-3 (dark grey). A truncation mutant was also generated after S865 (red), thereby lacking most of the C-tail.

### 3.2.2 Cell surface Expression

The functional response of CaSR is dependent, in part, on its level of expression at the cell surface. Therefore expression of the four mutants was assessed and compared to WTCaSR by transiently transfecting HEK-293 cells with FLAG-tagged constructs and probing with an anti-FLAG antibody (Figure 3.3). Total protein was assessed in cell populations fixed with methanol while fixation with 4% PFA was used to assess cell surface expression (refer to
experimental procedures section 2.3.4 and 2.7.1 [172]). The vector only background was subtracted from the raw A<sub>450</sub> readings, and the readings were normalised to total WT expression levels.

Of the total WT protein expressed in the cell population, 60% was expressed at the cell surface. All four mutants were expressed in HEK-293s and trafficked to the cell surface with their total and cell surface expression not significantly different from WTCaSR as tested by ANOVA.

![Figure 3.3 Effect of iL mutations and C-tail truncation on CaSR expression](image)

**Figure 3.3** Effect of iL mutations and C-tail truncation on CaSR expression
HEK-293 cells transiently transfected with FLAG-tagged constructs were labelled with anti-FLAG antibody after fixation with 4% PFA or methanol to measure cell surface or total expression, respectively (n=4; error bars represent ± SEM).

### 3.2.3 Functional analysis

I next tested the impacts of the four mutants expressed transiently in HEK-293 cells on changes in various intracellular metabolites in response to elevated Ca<sup>2+</sup> (0.5-30 mM). These included IP<sub>1</sub>, Ca<sup>2+</sup>, ERK1/2 and cAMP.
3.2.3.1 IP₁ accumulation

Mutants were transiently transfected in HEK-293 cells and IP₁ accumulation was assessed by a homogeneous time-resolved FRET (HTRF) assay where introduced donor labelled IP₁ competed with endogenous IP₁ to bind the acceptor labelled IP₁ antibody (experimental procedures sections 2.4 and 2.7.4). After baseline IP₁ were subtracted, the results were expressed as fold-change with respect to control (Ca²⁺₀.5 mM) (Figure 3. 4, Table 3. 2).

HEK-293 cells transfected with WTCaSR responded to elevated Ca²⁺₀ with increased IP₁ accumulation. At a maximal [Ca²⁺] of 20 mM, a 3.1 ± 0.6 fold-increase in IP₁ was observed (Table 3. 2). In cells expressing F706A, a 1.3 ± 2.6 fold-increase was observed, whereas in cells expressing E803A or the CaSR₈₆₆X truncation mutant a similar 1.0 ± 0.7 fold change was observed in response to stimulation with 20 mM Ca²⁺₀. These responses were ~30% of WT. L797A expressing cells exhibited an even greater impairment in IP₁ accumulation at 20 mM Ca²⁺₀ with only a 0.8 ± 0.1 fold increase from baseline, which was ~25% of the WT response. The maximal responses of all four mutants were significantly impaired compared to WT (p<0.001; 2-way ANOVA).
Figure 3.4 Impacts of CaSR mutants on $\text{Ca}^{2+}$ stimulated IP$_1$ accumulation in transiently transfected HEK-293 cells.

An HTRF assay was used to measure IP$_1$ accumulation of HEK-293 cells transiently transfected with WTCaSR, or CaSR mutants of il-2, il-3 or the C-tail. Donor-labeled IP$_1$ competed with endogenous IP$_1$ to bind acceptor-labelled anti-IP$_1$ antibody ($n=4$-5; error bars represent ± SEM).

Table 3.2 Impacts of il mutations and C-tail truncation on CaSR mediated IP$_1$ stimulation in transiently transfected HEK-293 cells.

Raw F$_{615}$/F$_{665}$ data was expressed as fold-change with respect to control (WTCaSR IP$_1$ reading at 0.5 mM $\text{Ca}^{2+}$). EC$_{50}$ for $\text{Ca}^{2+}$ and the E$_{\text{max}}$ (fold-change) for IP$_1$ stimulation were estimated by fitting the Hill equation to response data ($p<0.001$ compared to WTCaSR; NF-no accurate fit could be obtained by curve fitting).

<table>
<thead>
<tr>
<th></th>
<th>EC$_{50}$ for $\text{Ca}^{2+}$ (mM)</th>
<th>E$_{\text{max}}$ (fold-change)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6.6 ±1.6</td>
<td>3.1 ± 0.6</td>
<td>5</td>
</tr>
<tr>
<td>F706A</td>
<td>16.1 ± 3.3</td>
<td>1.3 ± 2.6$^a$</td>
<td>5</td>
</tr>
<tr>
<td>L797A</td>
<td>NF</td>
<td>0.8 ± 1.0$^a$</td>
<td>5</td>
</tr>
<tr>
<td>E803A</td>
<td>9.6 ± 3.3</td>
<td>1.0 ± 0.3$^a$</td>
<td>5</td>
</tr>
<tr>
<td>CaSR$^{866X}$</td>
<td>NF</td>
<td>1.0 ± 0.7$^a$</td>
<td>5</td>
</tr>
</tbody>
</table>
No significant difference in the Ca\(^{2+}\)-sensitivity was observed for the mutants when compared to one another or when compared to WT.

Since IP\(_1\) is generated by the action of phosphomonoesterases on Ins 1,4,5-P\(_3\) and Ins 1,4-P\(_2\) downstream of G\(_{q11}\)-PI-PLC signalling, these findings suggested that the three iL residues studied and the CaSR C-tail play an important role in G\(_{q11}\) signalling. To further investigate this, we tested the impact of these mutations on Ca\(^{2+}\)\(_i\) mobilisation, also downstream of G\(_{q11}\) signalling.

### 3.2.3.2 Impact of CaSR iL-2, iL-3 and C-tail mutants on Ca\(^{2+}\)\(_i\) mobilisation in HEK-293 cells

HEK-293 cells prepared on glass coverslips were transiently transfected with either WTCaSR or one of the four mutants: F706A, L797A, E803A or CaSR\(_{866}\)X. After transfection for 48 hours the cells were loaded with 5 \(\mu\)M Fura 2-AM for 90 min and then exposed to increasing [Ca\(^{2+}\)\(_o\)] (Experimental protocols 2.5.1). Changes in Ca\(^{2+}\)\(_i\) were monitored using fluorescence microscopy. The oscillatory responses collected over periods of 3 min per treatment were, using the F\(_{340}\)/F\(_{380}\) ratios as described in experimental procedures 2.5.2, integrated to permit the generation of Ca\(^{2+}\)\(_o\) concentration-response relationships (Figure 3.5; Table 3.3). The method used for integrating the response is described in greater detail in experimental procedures 2.7.2.
Figure 3.5 Impacts of iL-2, iL-3 mutations and C-tail proximal truncation: Ca\(^{2+}\)\(_o\) concentration-response analysis for Ca\(^{2+}\)\(_i\) mobilisation
Fura 2-AM loaded HEK-293 cells, transiently transfected with WTCaSR or one of the four CaSR mutants, were exposed to increments of Ca\(^{2+}\)\(_o\) and \(F_{340}/F_{380}\) responses were integrated to generate concentration-response curves (n=4-9; error bars represent ± SEM).

Table 3.3 Impact of iL-2, iL-3 mutations and CaSR C-tail truncation on Ca\(^{2+}\)\(_i\) mobilisation in transiently transfected HEK-293 cells.

<table>
<thead>
<tr>
<th></th>
<th>(\text{EC}_{50}) (mM)</th>
<th>(\text{E}_{\text{max}})</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.9 ± 0.6</td>
<td>13.6 ± 1.2</td>
<td>7</td>
</tr>
<tr>
<td>F706A</td>
<td>5.5 ± 0.8</td>
<td>8.4 ± 0.9(^a)</td>
<td>7</td>
</tr>
<tr>
<td>L797A</td>
<td>6.8 ± 1.4</td>
<td>5.9 ± 1.0(^b)</td>
<td>8</td>
</tr>
<tr>
<td>E803A</td>
<td>3.0 ± 0.6</td>
<td>7.7 ± 0.6(^b)</td>
<td>7</td>
</tr>
<tr>
<td>CaSR(_{865X})</td>
<td>2.3 ± 7.4</td>
<td>0.8 ± 0.6(^b)</td>
<td>4</td>
</tr>
</tbody>
</table>
The WtCaSR induced Ca\(^{2+}\)\(_o\) concentration-dependent Ca\(^{2+}\)\(_i\) mobilisation with an EC\(_{50}\) for Ca\(^{2+}\)\(_o\) of 2.9 ± 0.6 mM and an E\(_{\text{max}}\) of 13.6 ± 1.2 IFRU. Removal of the C-tail in CaSR\(_{866X}\) transfected cells abolished the CaSR Ca\(^{2+}\)\(_i\) mobilisation response. In addition there were significant impairments in the E\(_{\text{max}}\) for F706A, L797A and E803A of 8.4 ± 0.9, 5.9 ± 1.0 and 7.7 ± 0.6, respectively (p<0.01), which were ~50% of the WT response.

Interestingly, the impact of the iL-2 and iL-3 mutants were less marked on, Ca\(^{2+}\)\(_i\) mobilisation response than IP\(_1\) accumulation, although both IP\(_1\) accumulation and Ca\(^{2+}\)\(_i\) mobilisation were abolished in HEK-293 cells that were transiently transfected with CaSR\(_{866X}\). This is consistent with the role of the iL residues and the CaSR C-tail in G\(_q/11\) mediated responses seen in IP\(_1\) accumulation.

We next examined the roles of the iL-2, iL-3 residues and proximal C-terminus in signalling pathways under the control of other G-proteins.

### 3.2.3.3 ERK1/2 Phosphorylation

Phosphorylation of ERK1/2 occurs in response to Ca\(^{2+}\)\(_o\)-dependent CaSR activation in HEK-293 cells and is downstream of G\(_q/11\), G\(_i/o\) and G\(_{12/13}\). The impacts of these mutants when transiently transfected in HEK-293 cells on ERK1/2 phosphorylation were examined in a FRET based Alphascreen assay as described in experimental procedures sections 2.6 and 2.7.5 (Figure 3, 6, Table 3.4). After baseline subtraction, the results were expressed as fold-changes with respect to the control WTCaSR ERK1/2 phosphorylation at 0.5 mM Ca\(^{2+}\)\(_o\).

The results show that in HEK-293 cells expressing WTCaSR, Ca\(^{2+}\)\(_o\) induced concentration-dependent increases in ERK1/2 phosphorylation levels 12-fold above baseline with an EC\(_{50}\) for Ca\(^{2+}\)\(_o\) of 2.4 mM (Table 3.4). The truncated CaSR C-tail abolished ERK1/2
phosphorylation. In addition the three point mutations in IL-2 and IL-3 significantly reduced the $E_{\text{max}}$ values to 2-fold or less with respect to WTCaSR at 0.5 mM $\text{Ca}^{2+}$ (p<0.001, ANOVA).

Figure 3. 6 Impacts of various CaSR mutations on $\text{Ca}^{2+}$-stimulated ERK1/2 phosphorylation in transiently transfected HEK-293 cells

Donor and acceptor labelled primary and secondary antibodies, respectively, were used to label phosphorylated ERK1/2 protein in cell lysates of $\text{Ca}^{2+}$-stimulated cells (n= 3-8; error bars represent ± SEM).
Table 3.4 Impacts of iL-2, iL-3 mutations and C-tail truncation on ERK1/2 phosphorylation in transiently transfected HEK-293 cells

The EC\textsubscript{50} for Ca\textsuperscript{2+}o and E\textsubscript{max} values (± standard error) of fold change for ERK1/2 phosphorylation were obtained by fitting the Hill equation to the experimental data (NF-no fit could be obtained by curve fitting; a (p<0.001) when compared to WTCaSR).

<table>
<thead>
<tr>
<th>EC\textsubscript{50} for Ca\textsuperscript{2+}o (mM)</th>
<th>E\textsubscript{max} (fold change)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.4 ± 0.3</td>
<td>5</td>
</tr>
<tr>
<td>F706A</td>
<td>1.4 ± 1.4</td>
<td>7</td>
</tr>
<tr>
<td>L797A</td>
<td>1.1 ± 1.3</td>
<td>8</td>
</tr>
<tr>
<td>E803A</td>
<td>1.8 ± 0.6</td>
<td>8</td>
</tr>
<tr>
<td>CaSR\textsubscript{855-X}</td>
<td>NF</td>
<td>8</td>
</tr>
</tbody>
</table>

The data demonstrate that iL-2, iL-3 residues as well as the C-tail are important for coupling to pERK1/2 as well as PI-PLC.

### 3.2.3.4 Ca\textsuperscript{2+}o-stimulated cAMP inhibition

We next examined the impact of the iL-2, iL-3 mutants and C-tail truncation on CaSR-mediated suppression of cAMP levels, which is dependent on G\textsubscript{i/o}-induced suppression of adenylyl cyclase [115]. HEK-293 cells were transiently co-transfected with a FRET cAMP reporter construct and one of the four CaSR mutants as described in experimental procedures 2.5.3. After transfection for 48 hours the cells were exposed to 1 μM forskolin to elevate intracellular cAMP levels and then exposed to increments in Ca\textsuperscript{2+}o. cAMP levels were monitored by fluorescence microscopy. The results were normalized to forskolin-stimulated cAMP levels as 100% after baseline subtraction (Figure 3.7, Table 3.5).
Figure 3.7 Impacts of IL-2, IL-3 mutations and C-tail truncation on Ca^{2+}_o-mediated suppression of 1 μM forskolin-stimulated cAMP levels in transiently transfected HEK-293 cells. A FRET-EPAC reporter construct measured cAMP levels in a real-time, live-cell fluorescence assay (n=3; error bars represent ± SEM).
Table 3.5 Summary of Ca\(^{2+}\)o-sensitivities and maximal response values for WT CaSR and CaSR mutants on intracellular cAMP levels

<table>
<thead>
<tr>
<th></th>
<th>IC(_{50}) for Ca(^{2+})o (mM)</th>
<th>E(_{\text{max}}) (% Forskolin-stimulated cAMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.7 ± 0.2</td>
<td>31 ± 3.2</td>
</tr>
<tr>
<td>F706A</td>
<td>NF</td>
<td>NF</td>
</tr>
<tr>
<td>L797A</td>
<td>4.6 ± 1.3</td>
<td>97 ± 3.7(^b)</td>
</tr>
<tr>
<td>E803A</td>
<td>0.8 ± 0.2</td>
<td>47 ± 5.4(^a)</td>
</tr>
<tr>
<td>CaSR(_{865X})</td>
<td>1.5 ± 0.5</td>
<td>47 ± 3.8(^a)</td>
</tr>
</tbody>
</table>

F\(_{\text{CFP}}\)/F\(_{\text{YFP}}\) ratios for the last minute of exposure to each Ca\(^{2+}\)o concentration were averaged and after baseline subtraction were expressed as % of the forskolin-stimulated cAMP response at 0.5 mM Ca\(^{2+}\)o. The data were fitted to a modified form of the Hill equation (NF-no fit could be obtained to the modified Hill equation; represents statistically significant \(^a\) (p<0.05), \(^b\) (p<0.001) when compared to WT; n=3).

HEK-293 cells transfected with vector alone did not show any changes in cAMP levels to elevated Ca\(^{2+}\)o (data not shown). On the other hand, HEK-293 cells transfected with the WT CaSR exhibited suppressed cAMP levels in response to elevated Ca\(^{2+}\)o, with an IC\(_{50}\) for Ca\(^{2+}\)o of 1.7 mM and a maximal 70% suppression of cAMP levels. F706A and L797A abolished Ca\(^{2+}\)o-dependent cAMP suppression. However, the response was maintained in HEK-293 cells transiently transfected with E803A or CaSR\(_{865X}\), although maximal suppression was apparently reduced to 50%. In addition, E803A exhibited enhanced Ca\(^{2+}\)o sensitivity with the IC\(_{50}\) for Ca\(^{2+}\)o dropping from 1.7 ± 0.2 to 0.8 ± 0.2, although this was not statistically significant.
3.3 Discussion

3.3.1 Mutational analysis of iL residues and C-tail truncation

3.3.1.1 The role of iL residues and C-tail in PLC signalling

Activation of $G_{q/11}$ stimulates PLC activity resulting in IP formation and $Ca^{2+}$ mobilisation [104, 173]. Since CaSR activation elicits changes in both intracellular metabolites, they are often assayed as readouts for coupling to $G_{q/11}$ [100].

In the present study, IP$_1$ accumulation and $Ca^{2+}$ mobilisation assays showed that residues in iL-2, iL-3 and the C-tail were required for PLC signalling by the CaSR. Mutating F706 in iL-2, L797 or E803 in iL-3, or truncating the receptor after S865 impaired responses to $Ca^{2+}$ by 40-95%, although total and surface expression were comparable to WT. These results confirm previous studies implicating F706, L797, E803 and the C-tail in $Ca^{2+}$-induced PLC signalling [42, 46].

3.3.1.2 The role of iL residues and C-tail in ERK1/2 phosphorylation

CaSR activation stimulates phosphorylation of the MAP kinase, ERK1/2, in CaSR-expressing HEK-293 cells [109]. The mechanism by which the CaSR mediates ERK1/2 phosphorylation is, however, complex being dependent on signalling pathways lying downstream of both $G_{q/11}$ and $G_{do}$ [100].

In the current study I investigated the role of residues in iL-2, iL-3 and the C-tail in ERK1/2 phosphorylation. All three iL residues studied as well as the CaSR C-tail contributed to ERK1/2 phosphorylation. Thus, mutating the iL residues or truncating the C-tail either markedly impaired phosphorylation of ERK by 80% in the cases of F706A, L797A or E803A
or abolished it altogether in the case of 866X. These findings demonstrate that residues required for PLC signalling are also required for ERK1/2 phosphorylation.

This is the first study that has that CaSR iL-2 and -3 residues mediate ERK1/2 phosphorylation. Previously, filamin binding to the C-tail abolished CaSR-mediated ERK1/2 phosphorylation [112].

The truncations of the CaSR C-tail have previously been examined for their effect on ERK1/2 phosphorylation. Zhang et al. (2005), concluded that the receptor truncated after residue 868 was able to phosphorylate ERK1/2 however the response to high Ca\textsuperscript{2+} concentration (5 mM) was only 50% of WT cells [88]. This is consistent with the current study, where removal of all but the three proximal residues of the CaSR C-tail effectively abolished ERK1/2 phosphorylation.

### 3.3.1.3 The role of iL residues and C-tail in cAMP inhibition

Suppression of cAMP levels arises at least in part from G\textsubscript{i/o} coupling to the Ca\textsuperscript{2+}-activated CaSR [100]. Unpublished data from our lab demonstrated that CaSR-mediated suppression of cAMP levels was downstream of pertussis toxin-sensitive G\textsubscript{i/o}, as pertussis toxin treatment abolished the response in CaSR expressing HEK-293 cells.

Of the four mutants studied, F706A and L797A abolished CaSR-mediated suppression of cAMP levels in forskolin-stimulated HEK-293 cells that were transiently transfected with either the WT or mutant CaSR constructs. In contrast, neither E803A nor 866X exhibited impaired responses. Thus, HEK-293 cells that were transiently transfected with these mutants retained Ca\textsuperscript{2+}-induced suppression of forskolin-stimulated cAMP levels. In both cases the maximal inhibitory effects were marginally impaired. The results suggest that F706 in iL-2
and L797 in iL-3 are required for G_{i/o} signalling whereas E803 in iL-3 and the C-tail are not. Further studies are needed to elucidate the exact role of E803 and the C-tail in G_{i/o} coupling investigating the impact of amino acid substitutions or C-tail extension, respectively in the case of CaSR_{866X} on cAMP inhibition.

3.3.2 Insights into G-protein coupling and selectivity

Based on their effects on different signalling pathways, the four mutants studied can be divided into two groups: those that are involved in G-protein coupling and those that are involved in G-protein selectivity.

3.3.2.1 Molecular determinants of G-protein coupling

We have identified two residues, F706 in iL-2 and L797 in iL-3, that appear to be involved in global G-protein coupling. Mutating these two residues significantly impaired CaSR-mediated signalling in all four assays tested i.e., IP_{1} accumulation, Ca^{2+}_{i} mobilisation, ERK1/2 phosphorylation and cAMP suppression. The results highlight the roles of residues in iL-2 and iL-3 in G-protein coupling to CaSR and demonstrate that interaction sites are required in both iL-2 and iL-3 for efficient G-protein coupling consistent with studies across the GPCR superfamily reporting that G-protein coupling is dependent on interaction sites located in different cytoplasmic domains including iL-2 and iL-3 [101].

3.3.2.2 Molecular determinants of G-protein selectivity

E803 in iL-3 and the C-tail contributed to G-protein selectivity. In particular, E803 and the CaSR C-tail are required for G_{q/11}-mediated PLC signalling, but not G_{i/o} mediated cAMP
inhibition. Although ERK1/2 phosphorylation is downstream of G\textsubscript{12/13}, it is not exclusively coupled to this pathway. Therefore, the effects of the iL residues and C-tail on G\textsubscript{12/13} coupling are inconclusive in this study. An assay exclusive for G\textsubscript{12/13} will need to be tested.

Therefore, for the first time this study identifies a residue (E803 in iL-3) and a domain (the C-tail) that discriminate between G\textsubscript{q/11} and G\textsubscript{i/o}. The results suggest that each G-protein to which the CaSR couples has unique molecular determinants for G-protein binding. This is consistent with previous studies that described roles for residues in iL-2 and the C-tail for G\textsubscript{q/11} but not G\textsubscript{i/o} or G\textsubscript{s} coupling in the class C GPCR, mGluR-1 [55, 168].

Although the exact roles of the iL residues and C-tail studied here are not yet clearly defined, it seems reasonable to speculate on the nature of the interactions. For example, they may directly recruit, bind and/or activate its G-protein partners [101]. Alternatively, they may play an indirect role by facilitating the adoption of an active conformation, or by supporting interactions with intermediary proteins such as members of the RGS family which act as adaptors between G-proteins and receptors [159-161].
Chapter 4
Chapter 4 Regulating dimer formation to investigate function of CaSR heterodimers

4.1 Introduction

4.1.1 Understanding dimerisation

In addition to the presence of large VFTDs, receptor dimerisation is also a common feature of class C GPCRs [174]. Receptors are present at the cell surface either as homodimers, like the CaSR and mGluRs, or heterodimers composed of different receptor subunits, like the GABA\textsubscript{B} and T1Rs.

Dimerisation has important implications for receptor expression and function, with each receptor domain being impacted differently. Very little is known about the role of dimerisation in CaSR signaling and how the different CaSR domains are affected. In order to further investigate dimerisation, I developed a system based on GABA\textsubscript{B} receptor heterodimerisation whereby CaSR wild-type and mutant heterodimers could be expressed at the cell surface.

4.1.2 Dimerisation of GABA\textsubscript{B} receptors

The GABA\textsubscript{B} receptors are class C GPCRs that are expressed in the CNS where they respond to the inhibitory neurotransmitter, $\gamma$-aminobutyric acid (GABA) and are functionally active heterodimers composed of two subunits, GABA\textsubscript{B1} and GABA\textsubscript{B2} [175].

Function of the recombinant GABA\textsubscript{B1} receptor is limited when the subunit is expressed on its own. However, upon co-expression with GABA\textsubscript{B2} subunit, receptor function is revealed
Further evidence for heterodimerisation was obtained in experiments in which the receptors were co-immunoprecipitated from purified cortical membranes and shown to co-localise in the rat brain [177]. GABA_B receptors were the first heterodimeric GPCRs to be described.

4.1.3 Molecular Basis of heterodimerisation in GABA receptors

Investigation of the mechanisms of heterodimerisation showed that the C-terminals of the GABA_B1 and GABA_B2 receptors were critical for heterodimerisation between the two receptor subtypes (Figure 4.1) [178] and a motif responsible for ER retention was identified in GABA_B1 which prevented the receptor from being expressed at the cell surface. The motif is a four amino acid sequence of the form, RXRR, which is also found in some ion channels and is involved in retrograde trafficking [179, 180]. Forward trafficking of the receptor is achieved when it heterodimerises with the GABA_B2 receptor by interactions between alpha helices in their C-tails. The dimerisation shields the ER retention motif in GABA_B1, and thus the heterodimeric complex is trafficked to the cell surface where it is functional [181].
Figure 4.1 \( \text{GABA}_B \) heterodimer expression system

The \( \text{GABA}_{B1} \) receptor (red) contains an ER retention signal (black) in its C-tail. When it heterodimerises with \( \text{GABA}_{B2} \) (blue), this signal is shielded allowing trafficking of the heterodimer to the cell surface.

4.1.4 Class C GPCRs and \( \text{GABA}_B \) chimeras to regulate receptor expression

Identifying the role of the C-tails in the \( \text{GABA}_B \) trafficking system has generated possibilities to study dimerisation in other class C GPCRs [79]. Generating chimeras with \( \text{GABA}_B \) receptors allows for the manipulation of receptor trafficking and expression at the cell surface, and has been used successfully to study various mGlu receptors (Figure 4.2) [37, 79, 97]. Like the CaSR, mGluRs can homodimerise. In these studies, the last 326 residues of the C-tail in mGluR-5 were replaced with the last 88 residues of the \( \text{GABA}_{B1} \) C-tail containing the ER retention motif (mG5C1), or of the last 180 residues of the \( \text{GABA}_{B2} \) C-tail containing the shielding region (mG5C2) [79]. Forward trafficking and cell surface expression of mG5C1 was thus dependent on dimerisation with mG5C2 in the same way that \( \text{GABA}_{B1} \) trafficking is dependent on \( \text{GABA}_{B2} \).
The C-tail of mGluR5 (residues H845-Stop1172) was replaced with either the C-tail of GABA$_{B1}$ (residues T872-Stop961), containing the ER retention motif to generate mG5C1, or the GABA$_{B2}$ tail (residues F760-Stop941) to generate mG5C2. The chimeras permitted mGluR-5 heterodimer expression at the cell surface [79].

4.1.5 CaSR CTD and expression

Although residues 865-1078 of the CaSR C-tail can be deleted without impairing cell surface expression, several C-tail regions are important for the regulation of expression [45-47]. Thus, an alpha helix is predicted between residues 877 and 891 and is required for efficient trafficking [46]. In addition, two arginine-rich motifs, that contribute to ER retention are located between residues 890-898 [84]. By containing elements for both forward trafficking and intracellular retention C-tail, homodimerisation of the CaSR is necessary for efficient cell surface expression.

The WTCaSR C-tail presents an obstacle to controlled expression of mutant heterodimers. Therefore, in order to study the subunit requirements of nutrient sensing and signaling in CaSR dimers, I generated CaSR-GABA$_{B}$ chimeras to control the expression of CaSR heterodimers at the cell surface in a manner analogous to that described previously for mGlu-GABA$_{B}$ constructs [79]. I expected that a CaSR-GABA$_{B1}$ (CaSR-B1) construct would be retained intracellularly, and only trafficked to the cell surface when co-expressed with a
CaSR-GABA$_{B2}$ (CaSR-B2) chimera. Although CaSR-B2 homodimers might also be expressed at the cell surface, I expected that the introduction of inactivating mutations into this construct would reduce the signalling activities of these receptors. In this way I set out to generate a system to study the requirements of CaSR function in mutant heterodimers containing mutations in one or both subunits.

4.2 Results

4.2.1 Generating CaSR-GABA$_{B1}$ chimeras

I first determined how much of the CaSR C-tail should be deleted to permit the GABA$_{B1}$ ER retention motif to exert a dominant effect on surface expression. To achieve this, four CaSR-GABA$_{B1}$ (CaSR-B1) chimeras were generated employing strategies described in experimental procedures section 2.1 (Figure 4.3).
Figure 4.3 Generating CaSR-GABA$_{B1}$ chimeras

(A) GABA$_{B1}$ tail residues 854-960 that were added to CaSR truncation mutants to generate CaSR-GABA$_{B1}$ chimeras. The ER retention signal is underlined.  (B) Four CaSR C-tail truncation constructs were made using PCR amplification to introduce a stop codon immediately after each of the residues highlighted in grey: S865, S875, R890 and S908.  (C) Schematic representation of CaSR-GABA$_{B1}$ constructs. Chimeras were generated by appending the final 107 residues of the mouse GABA$_{B1}$ tail (red, broken lines) to the truncated CaSR constructs utilising an endogenous or introduced Bam HI site.
4.2.1.1 *CaSR*<sub>908</sub>-B1

The CaSR<sub>908</sub>-B1 construct had been generated previously by Mr. Roy Chan. Briefly, PCR was used to amplify the GABA<sub>B1</sub> tail with a 5’ *Bam* HI and 3’ *Xba* I site. This fragment was then ligated into CaSR-pcDNA3.1 which had been digested with *Bam* HI and *Xba* I to replace the C-terminal 170 residues of the CaSR with the final 107 residues of the mouse GABA<sub>B1</sub> C-tail. DNA sequencing confirmed that the construct had been assembled successfully.

4.2.1.2 *CaSR*<sub>890</sub>-B1, *CaSR*<sub>875</sub>-B1 and *CaSR*<sub>865</sub>-B1

Initially I attempted to ligate a PCR-amplified GABA<sub>B1</sub> tail fragment into a pcDNA3.1 backbone with a PCR-amplified CaSR fragment truncated after codon Ser890 without success. Attempts at excising the GABA<sub>B1</sub> tail from the CaSR<sub>908</sub>-B1 construct by *Bam* HI and *Xba* I digestion also failed due to resistance of *Xba* I site to cleavage.

An alternative method was devised in which the CaSR<sub>908</sub>-B1 plasmid was digested with *Kpn* I and *Bam* HI, and the products separated by agarose gel electrophoresis. Two bands were observed, a ~ 3.0 kb band corresponding to the CaSR, and a ~6.0 kb band corresponding to the GABA<sub>B1</sub> tail-pcDNA3.1 fragment. The 6.0 kb band was excised and the DNA extracted and purified (refer to Experimental protocols 2.1.11). CaSR truncation products were ligated into this construct using the *Kpn* I and *Bam* HI sites.

Using the WT CaSR plasmid as a template, the first 2670 nucleotides of the CaSR were PCR amplified using a forward primer with a 5’ *Kpn* I site and a reverse primer which bound up to residue 890 (nt 2670) followed by a 3’ *Bam* HI site (Appendix I). This fragment was digested with *Kpn* I and *Bam* HI and ligated into the pcDNA3.1-GABA<sub>B1</sub> tail backbone. Following transformation of the ligation reaction, miniprep plasmid purifications were obtained of
selected colonies. Success of ligations was analysed by restriction digest and confirmed by DNA by sequencing.

CaSR_{875}-B1 and CaSR_{865}-B1 constructs were generated in a similar way. Briefly, PCR fragments with reverse primers designed to bind up to nucleotide 2595 or 2625, and possessing a 5’ Kpn I and 3’ Bam HI site, were ligated into the pcDNA3.1-GABA_{B1} backbone and transformed and purified as described above. Sequencing confirmed the generation of the CaSR_{890}-B1, CaSR_{875}-B1 and CaSR_{865}-B1 constructs. These constructs were used for functional analysis.

4.2.2  CaSR(FLAG)-B1

Truncated CaSR-B1 constructs containing the FLAG-tag were obtained in the same way as described above (section 4.2.1.2), however the CaSR(FLAG) plasmid replaced the WTCaSR as the template in PCR reactions. CaSR(FLAG)_{908}-B1, CaSR(FLAG)_{890}-B1, CaSR(FLAG)_{875}-B1 and CaSR(FLAG)_{865}-B1 were generated and confirmed by sequencing. These constructs were generated to analyse the expression of the CaSR-B1 chimeras.

4.2.3  Generating truncated CaSR constructs

CaSR truncated receptors were generated to control for the effect of truncation on CaSR expression and activity. PCR amplification was used to truncate CaSR at various residues in the C-tail. A single forward primer, designed to bind to the 5’ end of WTCaSR cDNA with a 5’ Kpn I site, was used in conjunction with a series of reverse primers designed to bind to a stretch of cDNA up to the truncation, followed by a stop codon and a 3’ Xba I site (Appendix I). The PCR amplified truncated CaSR cDNA was then digested with Kpn I and Xba I and
ligated into pcDNA3.1 digested with the same enzymes. DNA Sequencing confirmed the truncation and the presence of the stop codon in all constructs.

Truncated CaSR constructs (CaSRX) were generated in a similar way with CaSR(FLAG) to obtain CaSR(FLAG)X constructs to measure expression.

4.2.3.1 *Expression analysis of CaSR-GABA<sub>B1</sub> chimeras*

I first tested the effects of truncating the CaSR C-tail at four sites: 865, 875, 890 and 908 and appending the final 107 residues of the mouse GABA<sub>B1</sub> C-tail on total and cell surface expression. Truncated CaSR constructs in which the GABA<sub>B1</sub> C-tail was not appended were included as controls.

HEK-293 cells were plated in 96-well plates and transiently transfected with either FLAG-tagged WTCaSR, CaSRX, CaSR-B1 or the pcDNA3.1 vector. Following fixation with either 4% PFA for the measurement of cell surface expression, or 100% methanol for the measurement of total expression cells were labelled with anti-FLAG antibody, conjugated to HRP and processed as described in experimental protocols section 2.3.4. The results were corrected for the vector only control by subtraction and the raw data were then normalised to the level of total WT expression which was defined as 100% (Figure 4.4).

The WTCaSR, CaSRX and CaSR-B1 constructs were successfully expressed 48 h after transfection in HEK-293 cells. With respect to the WT control, 65% was at the cell surface. Interestingly, in the absence of the GABA<sub>B1</sub> C-tail, CaSR truncations at S908 or R890 significantly enhanced cell surface expression. On the other hand truncations after S875 or S865 had no significant effect on cell surface expression compared to WT. Furthermore, the
additions of the GABA\textsubscript{B1} tail to all four CaSR truncations markedly reduced the level of cell surface expression to \(~50\%) of their truncated counterparts (Figure 4.4).

![Graph showing % Total WTCaSR expression vs. Cell Surface expression for various CaSR truncation mutants](image)

**Figure 4.4** Effects of appending the GABA\textsubscript{B1} tail on total and cell surface expression of various CaSR truncation mutants

HEK-293 cells transiently transfected with FLAG-tagged wild-type CaSR, CaSR constructs truncated or appended with the GABA\textsubscript{B1} C-tail after the following residues: S865, S875, R890 or S908. Transfected cells were fixed with either 4\% PFA, for cell surface expression, or 100\% methanol, for total expression, and incubated with an anti-FLAG antibody in an expression ELISA. The results were normalised to total WT(FLAG) expression (statistically significant difference in mutant cell surface expression \(a\) (p< 0.05), \(b\) (p<0.01), \(c\) (p<0.001) when compared to WT; statistically significant difference in CaSR-B1 cell surface expression \(d\) (p<0.01) when compared to their CaSRX counterparts; n=3-11).

Truncations of the CaSR C-tail in the absence or the presence of the appended GABA\textsubscript{B1} C-tail had no significant effect on total expression when compared to WT.

4.2.3.2 *Functional analysis of CaSR-GABA\textsubscript{B1} chimeras*

To evaluate the effects of CaSR C-tail truncation, GABA\textsubscript{B1} C-tail addition and associated changes in cell surface expression on CaSR function, I examined Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}i
mobilisation of transiently transfected, HEK-293 cells loaded with Fura 2-AM as described in experimental procedures section 2.5.2. Wild-type CaSR transfected cells were used as a positive control and pcDNA3.1 vector only transfected cells were used as a negative control (Figure 4. 5). I hypothesised that reductions in cell surface expression of CaSR-B1 constructs would markedly impair receptor function.

Wild-type CaSR transfected HEK-293 cells responded at Ca\textsuperscript{2+}\textsubscript{o} threshold levels of around 2 mM. The responses took the form of low frequency (1-4 min\textsuperscript{-1}) Ca\textsuperscript{2+}\textsubscript{i} oscillations at low Ca\textsuperscript{2+}\textsubscript{o} and sustained elevations in Ca\textsuperscript{2+}\textsubscript{i} at higher Ca\textsuperscript{2+}\textsubscript{o} concentrations (4-6 mM). As expected, pcDNA3.1 transfected HEK-293 cells were unresponsive to Ca\textsuperscript{2+}\textsubscript{o} at concentrations up to 20 mM. The responses were integrated and after subtraction of vector-only background, concentration-response curves were generated for each of the CaSR mutants and chimeras. The resulting data were fitted to the Hill equation (Figure 4. 6, Table 4. 1). For the WT CaSR the EC\textsubscript{50} for Ca\textsuperscript{2+}\textsubscript{o} was 4.1 ± 0.2 mM and the E\textsubscript{max} was 10 ± 0.3 IFRU (refer to experimental procedures section 2.7.2).

The CaSR 909X truncation exhibited increased E\textsubscript{max} compared to the wild-type CaSR and the patterns of response at low Ca\textsuperscript{2+}\textsubscript{o} and high Ca\textsuperscript{2+}\textsubscript{o} were maintained. Appending of the GABA\textsubscript{B1} tail to CaSR\textsubscript{908} resulted in a significantly reduced the E\textsubscript{max} to around 70% of WT and 50% of CaSR\textsubscript{909X}.

Truncating the CaSR after R890 increased E\textsubscript{max}. However, Ca\textsuperscript{2+}\textsubscript{i} oscillations were impaired at low Ca\textsuperscript{2+}\textsubscript{o} concentrations, and Ca\textsuperscript{2+}\textsubscript{i} levels failed to plateau at Ca\textsuperscript{2+}\textsubscript{o} concentrations up to 20 mM resulting in an inability to accurately calculate an EC\textsubscript{50} or E\textsubscript{max}. Appending the GABA\textsubscript{B1} tail reduced E\textsubscript{max} compared to CaSR\textsubscript{891X} and also appeared to reduce Ca\textsuperscript{2+}\textsubscript{o} sensitivity. However, CaSR\textsubscript{890-B1} also failed to exhibit plateau Ca\textsuperscript{2+}\textsubscript{i} responses and attempts at fitting the data to the Hill equation resulted in unreliable estimates of E\textsubscript{max} and EC\textsubscript{50}.  

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In the case of 876X there was a drop in the number of responsive cells from around 90% for WTCaSR to around 50%. Reduced Ca\textsuperscript{2+}\textsubscript{o} sensitivity, but increased $E_{\text{max}}$ was observed in cells that were responsive. Furthermore, low frequency Ca\textsuperscript{2+}\textsubscript{i} oscillations at high Ca\textsuperscript{2+}\textsubscript{o} or sustained Ca\textsuperscript{2+}\textsubscript{i} increases were observed. Appending the GABA\textsubscript{B1} tail to the CaSR\textsubscript{876X} abolished Ca\textsuperscript{2+}\textsubscript{i} mobilisation at Ca\textsuperscript{2+}\textsubscript{o} concentrations up to 20 mM.

Removal of all but three residues of the CaSR C-tail in the 866X construct also abolished Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation. In addition, no response was observed in CaR\textsubscript{865-B1} transfected cells.
Figure 4.5 Representative Ca\(^{2+}\)\(_o\)-induced Ca\(^{2+}\)\(_i\) mobilisation responses in CaSR-B1 chimeras.

Responses from 10 regions of interest were measured as a ratio of emitted fluorescence at 340 nm/380 nm (F340/F380), from transiently transfected HEK-293 cells upon exposure to increasing increments of Ca\(^{2+}\)\(_o\) as indicated. HEK-293 cells were transiently transfected with (A) WT-CaSR, (B) pcDNA3.1, (C) CaSR\(_{939}\)X, (D) CaSR\(_{928}\)B1 (E) CaSR\(_{891}\)X, (F) CaSR\(_{890}\)B1, (G) CaSR\(_{876}\)X, (H) CaSR\(_{875}\)B1, (I) CaSR\(_{866}\)X, or (J) CaSR\(_{865}\)B1.
**Figure 4.6** Effect of appending the GABA$_{B1}$ tail on the function of various CaSR truncation mutants

Ca$^{2+}$i mobilisation responses of transiently transfected HEK-293 cells were integrated and fitted to the hill equation. HEK-293 cells were transfected as shown. Mean integrated responses from 3-4 independent experiments after vector only background was subtracted (n=3-8).

**Table 4.1** Effects of CaSR C-tail truncations on Ca$^{2+}$i mobilisation response in transiently transfected HEK-293 cells

 transiently-transfected HEK-293 cells were loaded with Fura 2-AM, exposed to increments of Ca$^{2+}o$ and the Ca$^{2+}$i mobilisation response monitored by fluorescence microscopy. EC$_{50}$ for Ca$^{2+}o$ and E$_{max}$ values (± standard error) were estimated by fitting the Hill equation to the concentration-response curves in GraphPad Prism (represent statistical significance a (p<0.05), b (p<0.001) when compared to WT; N.F. – no fit could be obtained; n=3-8).

<table>
<thead>
<tr>
<th></th>
<th>EC$_{50}$ for Ca$^{2+}o$ (mM)</th>
<th>E$_{max}$ (IFRU)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>4.1±0.2</td>
<td>10 ± 0.3</td>
</tr>
<tr>
<td>CaR$_{999}X$</td>
<td>3.0 ± 0.3</td>
<td>12.3 ± 0.5$^a$</td>
</tr>
<tr>
<td>CaR$_{891}X$</td>
<td>N.F.</td>
<td>N.F.</td>
</tr>
<tr>
<td>CaR$_{876}X$</td>
<td>7.5 ± 0.6</td>
<td>11.3 ± 0.8</td>
</tr>
<tr>
<td>CaR$_{866}X$</td>
<td>2.3 ± 1.3</td>
<td>0.8 ± 0.1$^b$</td>
</tr>
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</table>
Table 4. Effects of appending the GABA<sub>B1</sub> C-tail on Ca<sup>2+</sup><sub>i</sub>-induced Ca<sup>2+</sup><sub>i</sub> mobilisation mediated by CaSR C-tail truncation mutants

Transiently-transfected HEK-293 cells were loaded with Fura 2-AM, exposed to increments of Ca<sup>2+</sup><sub>o</sub> and the Ca<sup>2+</sup><sub>i</sub> mobilisation response monitored by fluorescence microscopy. EC<sub>50</sub> for Ca<sup>2+</sup><sub>o</sub> an E<sub>max</sub> values (± standard error) were calculated by fitting the Hill equation to the concentration-response curves in GraphPad Prism (represent statistical significance a (p<0.05) compared to WT; b (p<0.001) when compared to WT; c (p<0.001) when compared to truncated CaSR control; NF – no fit could be obtained; n=3-8)

<table>
<thead>
<tr>
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<th>EC&lt;sub&gt;50&lt;/sub&gt; for Ca&lt;sup&gt;2+&lt;/sup&gt;&lt;sub&gt;o&lt;/sub&gt; (mM)</th>
<th>E&lt;sub&gt;max&lt;/sub&gt; (IFRU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.1 ± 0.2</td>
<td>10 ± 0.3</td>
</tr>
<tr>
<td>CaR&lt;sub&gt;908&lt;/sub&gt;-B1</td>
<td>3.9 ± 0.2</td>
<td>6.9 ± 0.2&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CaR&lt;sub&gt;990&lt;/sub&gt;-B1</td>
<td>N.F.</td>
<td>N.F.</td>
</tr>
<tr>
<td>CaR&lt;sub&gt;875&lt;/sub&gt;-B1</td>
<td>N.F.</td>
<td>0.5 ± 0.5&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CaR&lt;sub&gt;865&lt;/sub&gt;-B1</td>
<td>7.9 ± 1.5</td>
<td>1.6 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>

The data indicate that appending the GABA<sub>B1</sub> tail to the CaSR C-tail impairs cell surface expression in all cases, however, only appendage after Ser875 abolished Ca<sup>2+</sup><sub>i</sub> mobilisation.

4.2.4 CaSR-GABA<sub>B2</sub> chimeras

CaSR-GABA<sub>B2</sub> chimeras were also generated to rescue expression of the CaSR-B1 construct to the cell surface. Based on previous work [79, 99] two chimeras were generated. Residues 760-941 of the human GABA<sub>B2</sub> C-tail were appended to CaSR truncation mutant 909X (Figure 4. 7).

A variant of this construct (CaSR-B2<sub>KKTNX</sub>) was also generated in which the GABA<sub>B2</sub> C-tail was truncated after residue 820 followed by the intracellular retention motif, KKTN inserted just prior to the stop codon. Based on a previous study [99], this amendment was made with the aim of suppressing cell surface expression of the GABA<sub>B2</sub> tailed chimera to reduce signalling that might have arisen from CaSR-B2 homodimers.
Figure 4. 7 Design of CaSR-B2 chimeric receptors

(A) Sequence of the last 182 amino acids of the human GABA<sub>B2</sub> receptor. The coiled-coil domain which masks the ER retention signal of GABA<sub>B1</sub> is underlined. (B) Sequence of the GABA<sub>B2</sub> C-tail truncated after the coiled-coil domain followed by the addition of the retention motif KKTN (red) (C) Schematic representation of CaSR-GABA<sub>B2</sub> (CaSR-B2) chimera in which the GABA<sub>B2</sub> tail is appended to the CaSR truncated after residue 908 in the C-tail and the CaSR-B2 chimera truncated in the GABA<sub>B2</sub> tail (CaSR-B2<sub>KKTN</sub>X).

4.2.4.1 Generating CaSR-B2

The first CaSR-GABA<sub>B2</sub> (CaSR-B2) chimeric receptor was generated by Mr. Roy Chan. Briefly, the last 182 amino acids of the human GABA<sub>B2</sub> C-tail were PCR amplified with a 5’ Bam HI site and a 3’ Xba I site. After digestion with Bam HI and Xba I, the PCR product was ligated into CaSR-pcDNA3.1 which had been digested with Bam HI and Xba I to replace the
last 170 residues of the CaSR with the GABA_{B2} C-tail. The successful construct was confirmed by DNA sequencing.

FLAG-tagged CaSR-B2 (CaSR(FLAG)-B2) was generated by digesting CaSR(FLAG)_{908} with Kpn I and Bam HI followed by agarose gel electrophoresis, extracting and purification of the CaSR(FLAG)_{908} fragment. The insert was then ligated into purified GABA_{B2} tail-pcDNA3.1 vector backbone which had also been purified after digestion with the same enzymes. Successful creation of the construct was confirmed by DNA sequencing.

### 4.2.4.2 Generating CaSR-B2_{KKTNX}

CaSR-B2 was used as the template for PCR amplification. A forward primer with a 5’ Kpn I site and a reverse primer encoding the truncation and retention motif followed by a 3’ Xba I site were used in the PCR reaction (refer to Appendix II for primer sequences). The PCR product was purified, digested with Kpn I and Xba I and ligated into the pcDNA3.1 vector backbone digested with the same enzymes. To generate CaSR(FLAG)-B2_{KKTNX}, CaSR(FLAG)-B2 replaced CaSR-B2 as the template in the PCR reaction. DNA Sequencing confirmed the successful creation of both constructs.

### 4.2.4.3 Expression analysis of the CaSR-B2 chimeras

I first investigated the effects of replacing the last 107 amino acids of the CaSR C-tail with the last 182 amino acids of the human GABA_{B2} tail or the truncated GABA_{B2} tail (residues 760-820) extended by the ER retention motif KKTN on receptor expression using the ELISA protocols (experimental procedures section 2.3.4; Figure 4. 8). Because the GABA_{B2} tail does not contain any recognised ER retention motifs, it was hypothesised that appending the GABA_{B2} tail would have no effect on receptor expression. On the other hand the truncated
CaSR-B2\textsubscript{KKTN}X chimeric receptor was expected to impair cell surface expression compared due to the presence of the ER retention motif, KKTN.

The results demonstrated that appending the GABA\textsubscript{B2} tail or its variant had no significant effect on the level of total expression compared to WT or CaSR\textsubscript{909}X. However, although cell surface expression of CaSR-B2 was comparable to WT and CaSR\textsubscript{909}X, it was markedly impaired for CaSR-B2\textsubscript{KKTN}X to one-third of WT CaSR. Thus, surface expression of CaSR-B2\textsubscript{KKTN}X was only around 15%.

![Graph showing total and cell surface expression results for WT, CaSR\textsubscript{909}X, CaSR-B2, and CaSR-B2\textsubscript{KKTN}X](image)

**Figure 4.8 Total and cell surface expression results for WT CaSR and CaSR-B2 chimeric constructs**

HEK-293 cells were transiently transfected with either FLAG-tagged wild-type CaSR, CaSR\textsubscript{909}X or the chimeric receptors. The cells were incubated with HRP-conjugated anti-FLAG monoclonal antibody after fixation with either 4% PFA or 100% methanol to detect cell-surface expression or total expression, respectively (Experimental protocols 2.3.4). Results were expressed as a percentage of WT CaSR total expression (n=3-6; represent statistically significant differences in cell surface expression a (p<0.05), b (p<0.001) when compared to WT; c (p<0.001) when compared to CaSR-B2).
4.2.4.4 Functional Analysis of CaSR-B2 chimeras

Appendage of the GABA$_{B2}$ tail had no significant effect on Ca$^{2+}_{o}$-induced Ca$^{2+}_{i}$ mobilisation in Fura 2-AM loaded HEK-293 cells compared to WT or the CaSR$_{909X}$ truncation mutant (Figure 4. 9, Table 4. 3). Despite the significant reduction in the level of cell surface expression as noted above CaSR-B2$_{KKTNX}$ transfected cells retained Ca$^{2+}_{i}$ mobilisation response, with an EC$_{50}$ for Ca$^{2+}_{o}$ that was comparable to WT and an E$_{max}$ that was significantly reduced by around 50% compared to WT.
Figure 4. 9 Ca$^{2+}_o$-induced Ca$^{2+}_i$ mobilisation responses in HEK-293 cells expressing CaSR and CaSR-B2 chimeras

Ca$^{2+}_i$ mobilisation responses of transiently transfected HEK-293 cells were integrated and fitted to the Hill equation. HEK-293 cells were transfected with one of four constructs as shown. Integrated responses were plotted from 3-6 independent experiments after the vector-only background had been subtracted.

Table 4. 3 Ca$^{2+}_o$-sensitivities and the maximal responses of CaSR-B2 chimeric constructs

<table>
<thead>
<tr>
<th>Mutant</th>
<th>EC$^{50}$ for Ca$^{2+}_o$ (mM)</th>
<th>E$_{max}$ (IFRU)</th>
</tr>
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<tbody>
<tr>
<td>WTCaSR</td>
<td>3.7 ± 0.2</td>
<td>12 ± 0.3</td>
</tr>
<tr>
<td>CaSR$^{909}$X</td>
<td>3.0 ± 0.3</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td>CaSR-B2</td>
<td>2.7 ± 0.8</td>
<td>14 ± 1.4</td>
</tr>
<tr>
<td>CaSR-B2$^{KKTN}$X</td>
<td>3.9 ± 0.1</td>
<td>7 ± 0.2$^{a,b,c}$</td>
</tr>
</tbody>
</table>

4.2.5 Heterodimer trafficking

I next investigated whether CaSR-B2$^{KKTN}$X or CaSR-B2 would be co-chaperoned with CaSR-B1 or would be able to chaperone CaSR-B1, respectively, to the cell surface. Because CaSR-B2$^{KKTN}$X was retained intracellularly and had a reduced level of Ca$^{2+}_o$-induced signalling, I first investigated whether CaSR-B1 and CaSR-B2$^{KKTN}$X might be co-chaperoning to the cell surface.
4.2.5.1 *CaSR-B1 and CaSR-B2<sub>KKTNX</sub> co-chaperoning*

HEK-293 cells were transiently co-transfected with CaSR(FLAG)-B2<sub>KKTNX</sub> and one of the four different CaSR-B1 chimeras to measure expression of CaSR-B2<sub>KKTNX</sub> (Figure 4.10).

![Graph](image)

**Figure 4. 10 Test for co-chaperoning of CaSR-B2<sub>KKTNX</sub> with one of CaSR-B1 chimeric receptors**

FLAG-tagged CaSR-B2<sub>KKTNX</sub> constructs were co-transfected with un-tagged CaSR-B1 chimeras in HEK-293 cells to measure the expression of CaSR-B2<sub>KKTNX</sub> (n=3; significantly different cell surface expression compared to WT are represented by **p<0.01 and ***p<0.001).**

The impaired level of CaSR-B2<sub>KKTNX</sub> cell surface expression was not significantly enhanced upon co-expression with any of the CaSR-B1 constructs tested. The results indicate that CaSR-B2<sub>KKTNX</sub> is not a suitable candidate for expressing CaSR-B1/2 heterodimers at the cell surface.
4.2.5.2 CaSR<sub>875</sub>-B1 chaperoning by CaSR-B2

Since CaSR<sub>875</sub>-B1 exhibited significantly impaired cell surface expression as well as Ca<sup>2+</sup><sub>o</sub>-induced Ca<sup>2+</sup> mobilisation when expressed alone in HEK-293 cells, it was of interest to determine whether it would be chaperoned to the cell surface by CaSR-B2 dependent on an interaction between the GABA<sub>B1</sub> and GABA<sub>B2</sub> tails.

To test this expression of CaSR<sub>875</sub>-B1 was monitored in the presence of CaSR-B2 or CaSR<sub>909X</sub> (Figure 4. 11). Interestingly, an increase in the level CaSR<sub>875</sub>-B1 surface expression was observed upon co-expression with CaSR-B2 but not CaSR<sub>909X</sub>, which differed only by the presence of the appended GABA<sub>B2</sub> tail in the CaSR-B2 construct. This demonstrated that the trafficking of CaSR-B1 was enhanced not by a CaSR dependent interaction, but by a GABA<sub>B2</sub> C-tail interaction, most probably with the GABA<sub>B1</sub> tail, its recognised partner.

I also mutated the putative motif ER retention motif in CaSR<sub>875</sub>-B1 from RSRR to ASAR by site-directed mutagenesis (refer to Appendix II for primer sequences). The mutation significantly enhanced the level of cell surface expression compared to CaSR<sub>875</sub>-B1 demonstrating that the ER retention of the CaSR<sub>875</sub>-B1 construct required the retention motif in the GABA<sub>B1</sub> C-tail.
Figure 4. 11 Role of the GABA$_{B1}$ and the GABA$_{B2}$ interaction in cell surface chaperoning of CaSR-B1 by CaSR-B2
Total and surface expression of FLAG-tagged CaSR-B1 was examined in HEK-293 cells transfected in the absence or presence of CaSR-B2 or CaSR$^{909}$X. The expression of a mutant CaSR-B1 in which the ER retention motif RSRR was converted to ASAR was also investigated. The cells were incubated with HRP-conjugated anti-FLAG monoclonal antibody after fixation with either 4% PFA or 100% methanol to detect cell-surface expression or total expression, respectively (Experimental protocols 2.3.4) (n= 4-6; represent statistically significant differences with the indicated constructs $a$ (p<0.01), $b$ (p<0.001)).

I also investigated the expression of CaSR-B2 in the presence of CaSR$_{875}$-B1 (Figure 4. 12). A small reduction in the level CaSR-B2 cell surface expression was observed when it was co-expressed with CaSR$_{875}$-B1. A reduction in cell surface expression is consistent with the formation of CaSR$_{875}$-B1 and CaSR-B2 heterodimers with attendant reduction in the expression of CaSR-B2 homodimers at the cell surface.
Figure 4.12 The impact of CaSR<sub>875</sub>-B1 on the expression of CaSR-B2

Total and surface expression of FLAG-tagged CaSR-B2 was examined in HEK-293 cells transfected in the absence or presence of CaSR<sub>875</sub>-B1. The cells were incubated with HRP-conjugated anti-FLAG monoclonal antibody after fixation with either 4% PFA or 100% methanol to detect cell-surface expression or total expression, respectively (Experimental protocols 2.3.4) (n=3-7; represent statistically significant difference a (p<0.01) between cell surface expression of CaSR(FLAG)-B2 and CaSR<sub>875</sub>-B1 as measured by the t-test).
4.3 Discussion

4.3.1 Effect CaSR C-tail truncation on expression and function

The C-tail is important for CaSR trafficking, expression and function [20] however the nature of the relationships have not been clearly defined. In addition, the effect of mutant heterodimers on CaSR-mediated signalling is also poorly understood. In the current study, I generated four truncations of the C-tail after residues: 908, 890, 875 and 865 with the objective of generating CaSR-GABA_{B1} chimeric constructs in which the GABA_{B1} C-tail exerted a dominant ER retention effect sufficient to impair receptor expression.

Consistent with previous reports, no significant change in surface expression was observed in HEK-293 cells transiently transfected with CaSRs truncated after S875 or S865 [45-47]. On the other hand elevated cell surface expression was observed for receptors truncated after C-tail residues 908 and 890. This is consistent with a previous study that quantified the level of expression of truncated CaSR mutants [172]. An intracellular retention motif was previously identified between residues 890-898, and its deletion was reported to enhance receptor expression [172]. In the current study however, there were no significant difference between the level of expression of 909X, which retains this motif, and 891X which lacks it. Two alternative explanations seem plausible. Firstly, a region of the C-tail downstream of S908 may be required for intracellular retention. Alternatively, the C-tail may contain additional negative regulatory elements downstream of S908, deletion of which disables the effect of the proposed intracellular retention motif between 890-898 [84].

Enhanced receptor expression correlated with an increased E_{max} of Ca^{2+}_{o}-induced Ca^{2+}_{i} mobilisation in both CaSR_{909X} and CaSR_{891X}. The Ca^{2+}_{o} concentration response for CaSR_{876X} on the other hand was comparable to WT and CaSR_{866X} failed to respond to Ca^{2+}_{o} at concentrations up to 20 mM consistent with previous studies demonstrating that the C-tail...
is required for PLC signalling [46, 47]. CaSR<sub>891</sub>X and CaSR<sub>876</sub>X exhibited impaired Ca<sup>2+</sup><sub>i</sub>-induced Ca<sup>2+</sup><sub>i</sub> oscillations in was observed. For CaSR<sub>876</sub>X this is consistent with the idea that the primary PKC phosphorylation site at T888 is required for Ca<sup>2+</sup><sub>i</sub> oscillations [182]. The impairment in Ca<sup>2+</sup><sub>i</sub>-induced Ca<sup>2+</sup><sub>i</sub> mobilisation in oscillations is more surprising for CaSR<sub>891</sub>X since this receptor retains T888. The results suggest that a C-tail containing only two residues after T888 is insufficient for efficient receptor phosphorylation and requires investigation in future studies. This idea is supported by the observation that appending the GABA<sub>B1</sub> C-tail to CaSR<sub>890</sub> permitted Ca<sup>2+</sup><sub>i</sub>-induced Ca<sup>2+</sup><sub>i</sub> oscillations that were similar to WT.

4.3.2 GABA<sub>B1</sub> C-tail addition reduced expression

Appending the GABA<sub>B1</sub> C-tail to each of the four CaSR C-tail truncation mutants reduced the level of cell surface expression of the chimeric receptors to around half of the surface expression levels of their truncated counterparts consistent with the reported impact of the GABA<sub>B1</sub> tail on the level of surface expression of other class C GPCRS [79, 181]. However, the reduction in expression was less than expected. Residual expression may have been due to cell perforation arising from 4% PFA treatment resulting in a false high CaSR cell surface expression readout.

Although the level of surface expression was similar for each of the four CaSR-B1 chimeras, the functional impact was dependent on the length of the CaSR C-tail remaining. Thus, CaSR<sub>908</sub>-B1 and CaSR<sub>890</sub>-B1 were both functional but had impaired efficacy compared to CaSR<sub>909</sub>X and CaSR<sub>891</sub>X, respectively, likely due to the reduction in membrane expression.

On the other hand, truncation at 876X exhibited complete loss of receptor function in association with markedly impaired surface expression (Figure 4. 4, Figure 4. 5). This shows
that the receptor density threshold required for CaSR-mediated PLC signalling is dependent, at least in part, on the length of the CaSR C-tail.

4.3.3 Addition of GABA\textsubscript{B2} variants to CaSR\textsubscript{908}

The last 182 amino acids of the GABA\textsubscript{B2} C-tail were appended after CaSR residue 908, to generate a CaSR-B2 chimera with expression and function that were expected to resemble that of CaSR\textsubscript{909}X, consistent with studies in which the GABA\textsubscript{B2} tail had no effect on receptor expression or function when appended to mGluR-5 [79].

In an attempt to regulate the surface expression of a CaSR-B2 chimeric receptor, the GABA\textsubscript{B2} tail was prematurely truncated after residue 820 and the ER retention motif, KKTN was added (Figure 4. 7). This was previously shown to impair surface expression and to abolish the function of mGluR5- GABA\textsubscript{B2} homodimers [99]. The modification markedly impaired cell surface expression of CaSR-B2\textsubscript{KKTN}X to around 30% of the WTCaSR (Figure 4. 8), and less than a 25% of CaSR\textsubscript{909}X and CaSR\textsubscript{908}-B2. Surprisingly, however, CaSR-B2\textsubscript{KKTN}X retained function with efficacy suppressed to around 50% of WT and no apparent change in the EC\textsubscript{50} for Ca\textsuperscript{2+} (Figure 4. 9, Table 4. 1). The current findings support a previous report where CaSR C-tail residues 909-1078 were shown to negatively modulate PLC and removal of this region promoted PLC activity even when cell surface expression was significantly impaired [46].

Co-expression of CaSR-B2\textsubscript{KKTN}X with any of the four CaSR-B1 chimeras had no effect on its surface expression. A possible explanation is that the 46 residue CaSR C-tail present in the CaSR-B2\textsubscript{KKTN}X construct was too long or that secondary structures in the C-tail prevented the GABA\textsubscript{B1} and modified GABA\textsubscript{B2} tail from successfully interacting.

The CaSR\textsubscript{875}-B1 construct was selected from the CaSR-B1 chimera library for the analysis of heterodimer formation. It exhibited impaired function in association with reduced cell surface
expression. The CaSR-B2 chimeric receptor was selected for these experiments in preference to CaSR-B2KX since none of the CaSR-B1 chimeras enhanced expression of the latter.

4.3.4 CaSR-B1 and CaSR-B2 heterodimers

Analysis of surface expression demonstrated that impaired expression of CaSR_{875}-B1 was due to the ER retention motif of the GABA_{B1} C-tail. Its cell surface expression was enhanced when co-expressed with CaSR-B2 in a manner that was dependent on the interaction of the GABA_{B1} and the GABA_{B2} C-tails (Figure 4. 11). Furthermore, CaSR-B2 surface expression was slightly reduced when co-expressed with CaSR_{875}-B1 (Figure 4. 12) consistent with the notion of GABA_{B1} and GABA_{B2} heterodimerisation. Further work is required to confirm the existence of heterodimers e.g. by co-immunoprecipitation or FRET [79]. The results demonstrate that the GABA_{B} trafficking system can be applied to the CaSR as was previously applied to mGluR-5 [79, 183].

This system can be used to study the function of WT-mutant heterodimers since the CaSR_{875}-B1 receptor is not delivered to the cell surface and any function from CaSR-B2 homodimers present at the cell surface can be abolished by the introduction of inactivating mutations. As such any response will be from WT-mutant heterodimers present at the cell surface. Thus surface expression of CaSR_{875}-B1 is dependent on trafficking by CaSR-B2 and function of mutant heterodimers is dependent on CaSR_{875}-B1 and CaSR_{B2} heterodimers at the cell surface. A limitation of this system is that it is useful for studying loss-of-function mutations. The future study of gain-of-function mutations will require the development of a system in which cell surface expression of CaSR-B2 is dependent on CaSR-B1.
Chapter 5
Chapter 5  Minimum subunit requirements for nutrient sensing and signalling

5.1 Introduction

5.1.1 Dimerisation in biology

Biological systems are comprised of networks of proteins interacting with each other. Often these proteins will self associate to form dimers and higher order oligomers. Specifically, homodimerisation is a common phenomenon in living systems with more than a quarter of characterised enzymes existing as homodimers. This suggests it is sufficiently advantageous to be under such high selective pressure [184].

Indeed, by increasing the number of active or ligand-binding sites, dimerisation allows for co-operativity between different binding sites and also facilitates protein activity within a specific concentration range of ligand [174].

In some cases, dimer formation is the initial step in regulating protein activity. For example, Caspase-9, which exists in a monomeric, inactive form, forms homodimers as an initial step in the process of apoptosis. Dimerisation allows for Caspase-9 proteolytic activity allowing the cell to commit to programmed cell death [185].

Dimerisation is also an efficient way of generating large and complex protein structures [184]. It allows assembly of identical subunits to form functional complexes reducing the genome size. Furthermore, proteins synthesised from the same copy of mRNA tend to co-localise making it easier for proteins to homodimerise [184, 186]. Dimerisation reduces the
likelihood of proteins mis-folding because individual monomers are more likely to fold correctly compared to a single, large protein.

5.1.2 Dimerisation in GPCRs

Many GPCRs are known to form dimers. Some GPCRs are known to dimerise upon ligand binding like the chemokine receptor, CXCR4 [187], while other receptors require dimers for functional activity like the class C receptors [184]. Dimerisation might enhance the efficacy of downstream signalling by increasing the number of protein interaction sites [184, 188]. Interestingly, in the case of the serotonin type 4 receptors (5-HT₄R), only a single HHD is required for G-protein activation, but ligand binding in both subunits is required for full receptor activation [189]. This suggests that for different receptors the impact of dimerisation is different. Dimerisation may also regulate the trafficking of GPCRs to the cell surface [184, 190].

In the case of class C GPCRs, dimerisation is critical for receptor expression and function. Although the full role of dimerisation remains to be elucidated various structural and functional studies with the different receptors in this class have provided important insights [191].

The crystal structure of mGluR-1 showed that the VFTD dimer was the functional agonist binding unit at the cell surface [24]. Each VFTD was able to adopt an independent open/closed conformation based on whether it was bound to glutamate. Glutamate binding also re-orientated the two VFTDs relative to one another, which contributed to receptor activation. In the case of the GABAₓ receptors, heterodimerisation of GABAₓ₁and GABAₓ₂
increased agonist binding efficiency by up to ten-fold [176]. Thus, the co-operativity of ligand binding domains across the dimer interface is important for class C GPCR function.

The confirmation of the importance of dimerisation for function also raised many questions. Was closure resulting from agonist binding of both VFTDs necessary for receptor function, or was a single, closed VFTD sufficient? Would signal transmission between the VFTD and the HHD occur in cis, trans or both? Was a single HHD sufficient for G-protein coupling or were both HHDs required? In general, what was the contribution of each domain to the function of the dimeric receptors [79, 174]?

The system to regulate cell surface heterodimer expression by generating CaSR-GABA\textsubscript{B} chimeras described in the previous chapter, was designed with a specific aim: to understand the role of each domain in the functional CaSR dimer. A similar approach to studying mGluRs has revealed much about the function of domains in dimeric signaling.

5.1.3 Dimeric insights from chimeric mGluRs

Kniazeff et al. generated a similar system where the mGluR-5 C-tail was replaced with that of GABA\textsubscript{B\textsubscript{1}} (mG5C1) or GABA\textsubscript{B\textsubscript{2}} (mG5C2) [79]. One of three mutations in the VFTD that abolished agonist responses were introduced in mG5C2. When these mutant chimeras were co-expressed with mG5C1 chimeras, partial receptor activity was observed, suggesting that a single functional VFTD and the closed/open dimer formation was sufficient for receptor activation although the closed/closed VFTD dimer formation was necessary for full receptor activity. Furthermore, by introducing point mutations in iL-3 known to impair G-protein coupling they found that each VFTD was able to activate its own subunit’s HHD (cis activation), as well as the HHD of the other subunit (trans activation) [79]. A separate study
using the same system found that binding of the positive allosteric modulator (PAM), 3,3’ difluorobenzaldehyde, in a single HHD site was sufficient to achieve full receptor potentiation [183]. For mGlur-1, a single HHD was required for full coupling efficacy to G-proteins, but binding of the inverse agonist, 2-methyl-6-(phenylethynyl) pyridine, in both HHDs was necessary to inhibit receptor activity [192].

In contrast to the mGluRs the GABA_B receptors require only closure of the GABA_B1 VFTD to achieve full activity, but similarly to the mGluRs the single, GABA_B2 HHD is the prominent G-protein activator [174, 193]. Whether these findings also hold for the CaSR is, currently unknown.

In order to investigate the impact of CaSR dimerisation on agonist binding and G-protein activation requirements of PLC signalling, I selected five mutations to introduce in the heterodimer dimer expression system that might provide insight into CaSR dimer signalling.

### 5.1.4 Heterodimer expression system

The CaSR C-tail from residues 876-1078 was replaced with the last 107 amino acids of the GABA_B1 tail to generate CaSR-B1. The GABA_B1 C-tail contains an ER retention signal that sufficiently impairs cell surface trafficking of CaSR-B1 on its own to abolish receptor function.

A second chimera was generated in which the last 169 amino acids of the CaSR C-tail were replaced with the last 182 amino acids of the GABA_B2 C-tail to generate CaSR-B2. This addition had no significant effect on CaSR expression or function. However, when CaSR-B1 was co-expressed with CaSR-B2, its cell surface expression was enhanced due to the formation of heterodimers at the cell surface. I proposed to use this system to study the
impact of dimerisation on CaSR signalling by introducing impairing mutations in CaSR-B2 to reduce any functional noise from CaSR-B2 homodimers at the cell surface. Thus, any function observed would be from CaSR-B1 and CaSR-B2 heterodimers.

5.1.5 CaSR mutations in the VFTD
To investigate whether Ca\(^{2+}\) binding in one or two VFTDs was required for Ca\(^{2+}\)\(_o\)-dependent CaSR activation, I selected two naturally occurring inactivating mutations, G143E and R185Q to study in the CaSR-GABAB heterodimer expression system. Both these mutations reduce the receptor’s sensitivity to Ca\(^{2+}\)\(_o\). Patients with these mutations exhibit hypercalcaemia and hypocalciuria and often present with FHH or NSHPT [44, 86]. Although these mutations do not affect the glycosylation status of the receptor [86], initial studies showed that G143E exhibits reduced level of receptor expression compared to WT whereas R185Q had no effect on expression [86]. When expressed in HEK-293 cells G143E failed to elicit a PLC-mediated response to Ca\(^{2+}\)\(_o\) whereas R185Q had an \(E_{\text{max}}\) 36% of WT with a \(EC_{50}\) for Ca\(^{2+}\)\(_o\) that was markedly increased from around 4 mM to 26 mM [78]. These studies were performed on mutant CaSR homodimers [16, 86]. Co-expression of these mutants with the WT CaSR in HEK-293 cells demonstrated that the mutations may negatively affect the function of WT domains by forming heterodimers with reduced functional capacity [16].

5.1.6 A heterodimer system for investigating CaSR mutants
To further investigate the impact of these mutations on mutant-WT heterodimers, Bai et al. developed a system in which they co-expressed an inactive CaSR C-terminal truncation mutant 877X with either of the CaSR VFTD mutants, G143E or R185Q [78]. They found a
high level of heterodimerisation between G143E or R185Q and 877X as determined by co-immunoprecipitation. In addition, there was a recovery in the function of co-transfected cells with the Ca$^{2+}$ mobilisation $E_{\text{max}}$ increasing to 50% of WT function, substantially greater than observed in HEK-293 cells transfected with anyone of the three mutants alone. Interestingly, the EC$_{50}$ for Ca$^{2+}_o$ fell to 13 mM for R185Q/CaSR$_{877}X$ co-transfected cells and ~30 mM for G143E/CaSR$_{877}X$ co-transfected cells. They concluded that heterodimerisation was the underlying cause of the reconstitution of function observed in HEK-293 cells co-transfected with CaSR mutants and CaSR$_{877}X$, but that mutated domains may adversely affect the function of normal domains in the dimer [78].

This system was also used to investigate the impact of the inactivating iL-3 mutant, R795W, when co-expressed with CaSR$_{877}X$ or one of the VFTD mutants. An increase in the $E_{\text{max}}$ and enhanced sensitivity for Ca$^{2+}_o$ was observed [78]. When R795W was co-transfected with 877X, G143E or R185Q in HEK-293 cells, despite the partial recoveries in function observed, the proposed heterodimers exhibited significantly impaired Ca$^{2+}_o$-sensing and intracellular signalling when compared to WTCaSR. The study concluded that the dimers operated as coherent functional units, with the function of CaSR dependent on which mutants were co-expressed [78].

I selected the inactivating CaSR mutants, G143E and R185Q for the current study because they were known to impair VFTD function and had been studied previously in a CaSR heterodimer system and would therefore facilitate a comparison between the two systems.
5.1.7 CaSR iL signalling mutants

I was also interested in investigating the CaSR G-protein coupling requirements by studying iL mutant-WT heterodimers. Based on the work done in chapter 3, I selected three iL mutations to study in the CaSR-GABA\(_B\) heterodimer expression system: F706A in iL-2, L797A and E803A in iL-3. Although these mutants have not been reported in kindreds with disorders of calcium metabolism, they are known to impair Ca\(^{2+}\)o-stimulated PLC signalling without affecting receptor expression [42].
Chapter 5

Minimum subunit requirements for nutrient sensing and signalling

5.2 Results

5.2.1 Generation of CaSR mutants

The generation of CaSR-B1 and CaSR-B2 was described previously in section 4.3.2 and 4.3.3. Briefly, the first 2625 nucleotides of WTCaSR were PCR amplified with a 5’ Kpn I site and a 3’ Bam HI site as described in experimental procedures 2.1.8-2.1.9. The PCR product was digested and ligated into the pcDNA3.1 vector containing the GABA_B1 C-tail that had also been digested with Kpn I and Bam HI, to generate CaSR-B1 as described in experimental protocols 2.1.6-2.1.7. CaSR-B2 was generated by digesting WTCaSR with Kpn I and Bam HI and running the digest on the gel to separate the 2.7 kb band containing the first 2724 nucleotides from the remainder of the plasmid as described in experimental procedures 2.1.11. The 2.7 kb CaSR band was excised, the DNA extracted, purified and ligated into the pcDNA3.1 vector containing the GABA_B2 C-tail that had also been digested with Kpn I and Bam HI.

The two VFTD mutations, G143E and R185Q, and the three iL mutations, F706A, L797A and E803A (Appendix III), were individually introduced into WTCaSR, CaSR-B1 or CaSR-B2 vector backbones, with or without the FLAG epitope between residues 371 and 372. The mutations were introduced either by site-directed mutagenesis PCR or by digestion, DNA extraction and ligation by specific restriction enzymes (Table 5.1).
### Table 5.1 Generation of mutant CaSR-GABA<sub>B</sub> chimeras

Mutant chimeras were generated either by site-directed mutagenesis PCR (SDM) or by digestion and ligation (Lig.) at specific restriction sites. Where used, restriction sites have been specified.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Un-tagged</th>
<th>FLAG-tagged</th>
</tr>
</thead>
<tbody>
<tr>
<td>G143E</td>
<td>SDM</td>
<td>SDM</td>
</tr>
<tr>
<td>G143E-B1</td>
<td>Lig. (Kpn I &amp; Xho I)</td>
<td>SDM</td>
</tr>
<tr>
<td>G143E-B2</td>
<td>Lig. (Kpn I &amp; Bam HI)</td>
<td>SDM</td>
</tr>
<tr>
<td>R185Q</td>
<td>SDM</td>
<td>SDM</td>
</tr>
<tr>
<td>R185Q-B1</td>
<td>Lig. (Kpn I &amp; Xho I)</td>
<td>Lig. (Kpn I &amp; Xho I)</td>
</tr>
<tr>
<td>R185Q-B2</td>
<td>Lig. (Bam HI &amp; Xba I)</td>
<td>Lig. (Kpn I &amp; Xho I)</td>
</tr>
<tr>
<td>F706A</td>
<td>SDM</td>
<td>SDM</td>
</tr>
<tr>
<td>F706A-B1</td>
<td>-</td>
<td>SDM</td>
</tr>
<tr>
<td>F706A-B2</td>
<td>Lig. (Kpn I &amp; Bam HI)</td>
<td>SDM</td>
</tr>
<tr>
<td>L797A</td>
<td>SDM</td>
<td>SDM</td>
</tr>
<tr>
<td>L797A-B1</td>
<td>SDM</td>
<td>SDM</td>
</tr>
<tr>
<td>L797A-B2</td>
<td>SDM</td>
<td>SDM</td>
</tr>
<tr>
<td>E803A</td>
<td>SDM</td>
<td>SDM</td>
</tr>
<tr>
<td>E803A-B1</td>
<td>SDM</td>
<td>SDM</td>
</tr>
<tr>
<td>E803A-B2</td>
<td>Lig. (Kpn I &amp; Bam HI)</td>
<td>Lig. (Kpn I &amp; Xho I)</td>
</tr>
</tbody>
</table>

There was difficulty generating the F706A-B1 construct, so I used the F706A(FLAG)-B1 construct in all analyses on the impact of F706A on expression and function. The presence of the FLAG epitope has no affect on the function or expression of WTCaSR [16, 28].

#### 5.2.2 Expression analysis of CaSR-B1 & CaSR-B2 mutants

CaSR-B1 had impaired cell surface expression compared to WT, due to the presence of the ER retention motif in the GABA<sub>B</sub> C-tail. CaSR-B1 mutants also had impaired cell surface expression compared to WT. Interestingly a small enhancement in cell surface expression of
G143E-B1 compared to CaSR-B1 was observed. The level of total expression was not affected by any of the mutant constructs (Figure 5.1).

![Graph showing expression levels of various mutants](image)

**Figure 5.1 Expression of CaSR-B1 mutants**

Transiently-transfected HEK-293 cells with FLAG-tagged constructs were fixed with 4% PFA or 100% methanol to monitor cell surface and total expression, respectively. They were then incubated with HRP-conjugated anti-FLAG antibody and HRP activity was used to quantify expression (n=3-4; error bars represent SEM; represents statistically significant a (p<0.05) or b (p<0.001) differences compared to cell surface expression of the WTCaSR).

Introducing the point mutations into CaSR-B2 resulted in small increases in cell surface expression compared to WT, in a manner similar to CaSR-B2, whereas the total level of expression was not affected (Figure 5.1).

The expression results showed that none of the point mutations in the VFTD or the HHDs of the CaSR-B1 or CaSR-B2 constructs had a negative impact on either total or surface expression.
Figure 5.2 Expression of CaSR-B2 mutants
HEK-293 cells transiently-transfected with FLAG-tagged constructs were fixed with 4% PFA or 100% methanol to monitor cell surface and total expression, respectively. They were then incubated with HRP-conjugated anti-FLAG antibody and HRP activity was used to quantify expression (n=3-4; error bars represent SEM; represent statistically significant a (p<0.05), b (p<0.01) or c (p<0.001) changes in cell surface expression compared to WT).

5.2.3 Functional analysis of VFTD mutant-WT heterodimers

5.2.3.1 WT-G143E heterodimers
To test if the CaSR-GABA\textsubscript{B} sorting system would perform functionally as expected and to investigate whether a single active VFTD might be sufficient for \(Ca^{2+}\)-dependent signalling, I initially tested the impact of the G143E mutant. G143E was introduced into CaSR-B2 (G143E-B2) to reduce background \(Ca^{2+}\)-dependent \(Ca^{2+}\) mobilisation from CaSR-B2 homodimers which were expressed at high levels on the cell surface (Figure 5.2). It was also anticipated that G143E-B2 mutant would traffic the CaSR-B1 receptor to the cell surface enabling heterodimer expression and function.
As a control, G143E-B2 was also co-expressed with G143E-B1. We hypothesised that heterodimers arising from the co-expression of these receptors would be efficiently expressed but would exhibit markedly impaired receptor function comparable to that previously observed for G143E homodimers.

Transiently transfected HEK-293 cells were loaded with Fura 2-AM and exposed to increasing concentrations of $\text{Ca}^{2+}_o$ from a baseline level of 0.5 mM to a maximum level of 30 mM. The $\text{Ca}^{2+}_i$ mobilisation response was recorded and integrated, then corrected for vector only background response, to generate $\text{Ca}^{2+}_o$-response curves (Figure 5.3).

**Figure 5.3 Functional response of WT-G143E heterodimers**

The $\text{Ca}^{2+}_i$ mobilisation response of transiently transfected, Fura 2-AM loaded HEK-293 cells to $\text{Ca}^{2+}_o$ was integrated to generate concentration-response curves. (A) The effect of G143E in the full length CaSR, (B) the impact of G143E when introduced into CaSR-B2, and (C) the functional effect of a single G143E mutation in CaSR-B2 on the responses of either CaSR-B1 or G143E-B1 (n=3-6; error bars represent ± SEM).
Table 5. 2 Summary of Ca\(^{2+}\)o- sensitivity and maximal response G143E mutant dimers

The EC\(_{50}\) and E\(_{\text{max}}\) values ± standard errors were calculated by fitting the modified Hill equation to the integrated Ca\(^{2+}\)i mobilisation responses in GraphPad Prism. (represent significantly different a (p<0.01), b (p<0.001) when compared to WT; NF-no accurate fit could be obtained).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>EC(_{50}) for Ca(^{2+})o (mM)</th>
<th>E(_{\text{max}}) (IFRU)</th>
<th>Hill Coef.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT CaSR</td>
<td>4.3 ± 0.1</td>
<td>11 ± 0.2</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>G143E</td>
<td>N.F.</td>
<td>1.2 ± 0.1(^b)</td>
<td>N.F.</td>
</tr>
<tr>
<td>CaSR-B2</td>
<td>2.7 ± 0.7</td>
<td>14 ± 0.8(^a)</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>G143E-B2</td>
<td>2 ± 0.6</td>
<td>1.2 ± 0.4(^b)</td>
<td>N.F.</td>
</tr>
<tr>
<td>CaSR-B1 &amp; G143E-B2</td>
<td>11 ± 0.8(^a)</td>
<td>11 ± 0.7</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>G143E-B1 &amp; G143E-B2</td>
<td>N.F.</td>
<td>0.9 ± 0.3(^b)</td>
<td>N.F.</td>
</tr>
</tbody>
</table>

WT CaSR only transfected cells responded to increasing [Ca\(^{2+}\)o] by increasing Ca\(^{2+}\)i mobilisation, which plateaued at ~10 mM Ca\(^{2+}\)o. The EC\(_{50}\) for Ca\(^{2+}\)o was 4.3 mM, while the E\(_{\text{max}}\) value was around 11 IFRU. CaSR-B2-only transfected cells exhibited similar Ca\(^{2+}\)o-dependent responses to WT, but with a significantly higher efficacy, as reported previously (refer to chapter 4). Both WT and CaSR-B2 transfected cells were expected to express CaSR homodimers with neighbouring WT VFTDs.

Introducing G143E into either the full-length CaSR or the CaSR-B2 chimera, abolished Ca\(^{2+}\)i mobilisation (Figure 5. 3). These cells expressed homodimers in which both VFTDs were disabled by the G143E mutation. Despite normal or even increased levels of surface expression G143E-B2 transfected cells were functionally silent in assays of Ca\(^{2+}\)o-dependent Ca\(^{2+}\)i mobilisation.

As expected, co-expression of G143E-B1 with G143E-B2, to generate G143E mutated VFTD homodimers, abolished the response to Ca\(^{2+}\)o as expected. This demonstrated that the
presence and/or interaction of the GABA_B1/2 C-tails were not interfering with the CaSR response.

When CaSR-B1, which possessed a WT VFTD, was co-expressed with G143E-B2, which had a mutated VFTD, to generate WT/mutant heterodimers, significant recovery of function was observed. Since CaSR-B1 or G143E-B2 only transfected cells did not respond to Ca^{2+}_o, the functional response is deduced to have arisen from heterodimers composed of CaSR-B1 and G143E-B2. Although the efficacy of the response was similar to WT, the EC_{50} for Ca^{2+}_o was increased from 4.5 mM to 10 mM. These findings suggest that a single, functionally competent VFTD is sufficient to achieve maximal efficacy, but that two functional VFTDs are necessary for normal levels of Ca^{2+}_o sensitivity.

Testing G143E in the CaSR-GABA_B heterodimer expression system showed that the system could be used to test the impact of impairing mutations like, G143E, in mutant-WT heterodimers.

To see if two activated VFTDs were necessary for WT like Ca^{2+}_o sensitivity, or whether the result was dependent on the particular mutation selected, I next investigated the effect of another VFTD mutation, R185Q.

### 5.2.3.2 WT-R185Q heterodimers

HEK-293 cells transiently transfected with the CaSR mutant, R185Q, exhibited a significantly impaired E_{max} of Ca^{2+}_i mobilisation and the EC_{50} for Ca^{2+}_o dropped from 4 mM to 1.4 mM although there was uncertainty in the data impairing the calculation of error on the EC_{50} value (Figure 5.4, Table 5.3). Introducing the R185Q mutation in CaSR-B2 also
markedly impaired the $\text{Ca}^{2+}_o$-induced response. Indeed it was not possible to fit the data to the Hill equation to estimate either the $\text{EC}_{50}$ for $\text{Ca}^{2+}_o$ or $\text{E}_{\text{max}}$ values.

Cells transfected with CaSR-B1 & R185Q-B2, expressing WT-R185Q heterodimers however, had an $\text{E}_{\text{max}}$ value that was not significantly different from WT, and an $\text{EC}_{50}$ for $\text{Ca}^{2+}_o$ was significantly increased to around 12 mM. Co-expressing R185Q-B1 & R185Q-B2 to deliver mutant heterodimers in which neither VFTD was functionally competent mimicked the outcome of expressing R185Q alone, in which mutant homodimers are only expressed.

Figure 5.4 Functional response of WT-R185Q heterodimers
The $\text{Ca}^{2+}_i$ mobilisation response of transiently transfected, Fura 2-AM loaded HEK-293 cells to $\text{Ca}^{2+}_o$ was integrated to generate concentration-response curves. (A) The effect of R185Q in the full length CaSR, (B) the impact of R185Q when introduced into CaSR-B2, and (C) the functional effect of a single R185Q mutation in CaSR-B2 on the responses of either CaSR-B1 or R185Q-B1 (n=3-6; error bars represent ± SEM).
Table 5.3 Summary of Ca^{2+}_o-sensitivity and maximal response data for R185Q mutant dimers

EC_{50} and E_{max} values (±standard error) were calculated by fitting the modified Hill equation to the integrated Ca^{2+}_i mobilisation responses in GraphPad Prism. (Values are best-fit values ± SEM; represent significantly different a (p<0.05), b (p<0.0001) when compared to WT; c (p<0.0001) when compared to R185Q; NF-no fit could be obtained).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>EC_{50} for Ca^{2+}_o (mM)</th>
<th>E_{max} (IFRU)</th>
<th>Hill Coeff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT CaSR</td>
<td>4.4 ± 0.1</td>
<td>11 ± 0.2</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>R185Q</td>
<td>N.F.</td>
<td>0.5 ± 0.1</td>
<td>N.F.</td>
</tr>
<tr>
<td>CaSR-B1 &amp; R185Q-B2</td>
<td>12 ± 1.2^a</td>
<td>11 ± 0.4^c</td>
<td>4.4 fixed</td>
</tr>
<tr>
<td>R185Q-B1 &amp; R185Q-B2</td>
<td>N.F.</td>
<td>0.3 ± 0.1</td>
<td>N.F.</td>
</tr>
</tbody>
</table>

The CaSR-B1/CaSR-B2 heterodimers in which the CaSR-B2 construct contained either of the VFTD inactivating mutations G143E or R185Q exhibited E_{max} values comparable to the WT-CaSR, and an EC_{50} for Ca^{2+}_o that increased from around 4 mM to 11-12 mM. These results indicate that a loss of one functional VFTD impairs Ca^{2+}_o-sensitivity but not efficacy of signalling.

These findings suggest that two functional VFTDs are required for normal Ca^{2+}_o sensitivity but that signalling, at least in the form of Ca^{2+}_i mobilisation, may require only one HHD. Alternatively, one functional VFTD may be able to recruit both HHDs for G-protein dependent signalling.

To investigate this issue, I examined an additional three mutants in the WT-mutant heterodimer co-expression system in HEK-293, namely F706A, L797A and E803A. The impacts of these iL-2 and iL-3 mutants to impair CaSR-mediated G_{q/11} signalling has been previously described [42] and was re-examined for CaSR-dependent signalling via various signalling pathways in Chapter 4 of this thesis. Previously however, the impact of these mutations was examined under conditions in which both subunits were similarly affected in
mutant homodimers. What would happen to CaSR-mediated $\text{Ca}^{2+}_{i}$ mobilisation if only a single signalling impairing mutation was present in the CaSR dimer?

5.2.3.3 WT-F706A heterodimer signalling

The first of the three iL mutations investigated, F706A is located in iL-2. F706A homodimers significantly impaired the $E_{\text{max}}$ of the $\text{Ca}^{2+}_{o}$-activated CaSR-mediated $\text{Ca}^{2+}_{i}$ mobilisation response, although the EC$_{50}$ for $\text{Ca}^{2+}_{o}$ was not significantly different from WT (Figure 3. 5). As expected, similar data were obtained for HEK-293 cells transiently transfected with F706A-B2 (Figure 5. 5, Table 5.3).

Interestingly, in cells that were transfected with CaSR-B1 and F706A-B2 to express CaSR/F706A heterodimers at the cell surface, the $E_{\text{max}}$ value dropped from 11 IFRU for WT and around 8 IFRU for F706A or F706A-B2 expressing cells, to 5 IFRU. Also, the EC$_{50}$ for $\text{Ca}^{2+}_{o}$ markedly increased from 4 mM in WT expressing cells to 8 mM, although this was not significantly different from F706A or F706A-B2 expressing cells.

Surprisingly, there were no significant differences in the $\text{Ca}^{2+}_{o}$-induced curve fit parameters for $\text{Ca}^{2+}_{i}$ mobilisation responses for mutant heterodimers in which one or both HHDs bore the F706A mutation. This suggests that a single HHD is insufficient for WTCaSR signalling and that two functional HHDs are required to support signalling responses.
Figure 5.5 Ca\textsuperscript{2+}i mobilisation response of WT-F706A heterodimers

The Ca\textsuperscript{2+}i mobilisation response of transiently transfected, Fura 2-AM loaded HEK-293 cells to Ca\textsuperscript{2+}o was integrated to generate dose-response curves. (A) The effect of the F706A mutation in the full length CaSR, (B) the impact of F706A mutation when introduced into CaSR-B2, and (C) the functional effect of a single F706A mutation in the CaSR HHD dimer (n=3-5; error bars represent ± SEM).

Table 5.4 Summary of Ca\textsuperscript{2+}o sensitivity (EC\textsubscript{50}) and maximal response (E\textsubscript{max}) of F706A mutant dimers

The EC\textsubscript{50} and E\textsubscript{max} values ± standard error were calculated by fitting the modified Hill equation to the integrated Ca\textsuperscript{2+}i mobilisation responses in GraphPad Prism. (Values are best-fit values ± SEM; a represents significantly different (p<0.05), b (p<0.001), c (p<0.0001) compared to WT; d (P<0.0001) compared to F706A).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>EC\textsubscript{50} for Ca\textsuperscript{2+}o (mM)</th>
<th>E\textsubscript{max} (IFRU)</th>
<th>Hill Coeff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT CaSR</td>
<td>3.7 ± 0.2</td>
<td>11 ± 0.2</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>F706A</td>
<td>5.2 ± 0.5</td>
<td>7.5 ± 0.5\textsuperscript{b}</td>
<td>4.8 ± 2</td>
</tr>
<tr>
<td>F706A-B2</td>
<td>5.1 ± 0.3</td>
<td>8.4 ± 0.3\textsuperscript{a}</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>CaSR-B1 &amp; F706A-B2</td>
<td>8.2 ± 1.0\textsuperscript{a}</td>
<td>5.1 ± 0.4\textsuperscript{c,d}</td>
<td>3.8 ± 1.5</td>
</tr>
<tr>
<td>F706A-B1 &amp; F706A-B2</td>
<td>11 ± 17</td>
<td>10 ± 9</td>
<td>0.6 ± 0.7</td>
</tr>
</tbody>
</table>
5.2.3.4 Signalling from WT-L797A heterodimers

HEK-293 cells transiently transfected with the iL-3 mutants, L797A, L797A-B2, or co-transfected with L797A-B1 and L797A-B2 to express mutant homodimers at the cell surface, had $E_{\text{max}}$ values that were markedly reduced from 11 IFRU to around 4 IFRU (Figure 5.6, Table 5.5).

When HEK-293 cells were co-transfected with CaSR-B1 and L797A-B2, expressing WT/L797A heterodimers, the $E_{\text{max}}$ was markedly impaired by around 70%, similar to the L797A homodimers. However, there was no difference in the $Ca^{2+}_o$-sensitivity compared to WT in heterodimers in which one or both HHDs carried the L797A mutation. Interestingly, as observed for F706A (see previous section) WT-mutant heterodimers exhibited very similar $Ca^{2+}_o$-dependent $Ca^{2+}_i$ mobilisation responses indicating that one functional HHD was unable to compensate for the loss of a mutant HHD.

![Figure 5.6 Signalling from WT-L797A heterodimers](image)

The $Ca^{2+}_i$ mobilisation response of transiently transfected, Fura 2-AM loaded HEK-293 cells to $Ca^{2+}_o$ was integrated to generate concentration-response curves. (A) The effect of L797A mutation in the full length CaSR, (B) the impact of L797A when introduced into CaSR-B2 and (C) the functional effect of a single L797A mutation in CaSR-B2 HHD dimer ($n=3$-$4$; error bars represent ± SEM).
Table 5.5 Summary of Ca$^{2+}_o$-sensitivity and maximal response values of L797A mutant dimers

The EC$_{50}$ and E$_{max}$ values (± standard error) were calculated by fitting the modified Hill equation to the integrated Ca$^{2+}_i$ mobilisation responses in GraphPad Prism. Fixing the Hill coefficient to 1 allowed for curve fitting analysis of CaSR-B1 & L797A-B2 ($a$ represents significantly different (p<0.0001) when compared to WT).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>EC$_{50}$ for Ca$^{2+}_o$ (mM)</th>
<th>E$_{max}$ (IFRU)</th>
<th>Hill Coeff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT CaSR</td>
<td>3.7 ± 0.2</td>
<td>11 ± 0.2</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>L797A</td>
<td>6.1 ± 0.8</td>
<td>4.9 ± 0.5$^a$</td>
<td>4.2 ± 2.1</td>
</tr>
<tr>
<td>L797A-B2</td>
<td>4.7 ± 0.6</td>
<td>3.6 ± 0.3$^a$</td>
<td>4.8 ± 2.4</td>
</tr>
<tr>
<td>CaSR-B1 &amp; L797A-B2</td>
<td>1.7 ± 1.7</td>
<td>2.5 ± 0.3$^a$</td>
<td>0.6 ± 2.9</td>
</tr>
<tr>
<td>L797A-B1 &amp; L797A-B2</td>
<td>3.0 ± 0.4</td>
<td>2.4 ± 0.1$^a$</td>
<td>1.6 ± 0.3</td>
</tr>
</tbody>
</table>

5.2.3.5 Signalling from WT-E803A heterodimers

E803A was identified in the experiments described in chapter 3 as a unique mutation which when expressed as homodimers in HEK-293 cells selectively impaired CaSR-mediated IP$_1$ accumulation, ERK1/2 phosphorylation and Ca$^{2+}_i$ mobilisation, but had no effect on cAMP inhibition.

HEK-293 cells transiently transfected with E803A to express mutant homodimers had markedly impaired E$_{max}$ of Ca$^{2+}_o$-induced Ca$^{2+}_i$ mobilisation by around 30% compared to WT (Figure 5.7, Table 5.6). An impairment of 30-50% was also observed in HEK-293 cells transfected with E803A-B2 only, or co-transfected with E803A-B1 and E803A-B2, to express dimers mutated in both HHDS at the cell surface. No change in Ca$^{2+}_o$-sensitivity was observed in cells transfected with E803A or co-transfected with E803A-B1 and E803A-B2. Although a reduction in the EC$_{50}$ for Ca$^{2+}_o$ was observed in E803A-B2 transfected cells from about 4 mM to 2 mM, it was comparable to the Ca$^{2+}_o$-sensitivity for CaSR-B2 (Figure 5.7, Table 5.6).
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Minimum subunit requirements for nutrient sensing and signalling

Figure 5. 7 Signalling by E803A mutant dimers.
The Ca\textsuperscript{2+}\textsubscript{i} mobilisation response of transiently transfected, Fura 2-AM loaded HEK-293 cells to Ca\textsuperscript{2+}\textsubscript{o} was integrated to generate dose-response curves. (A) The effect of the E803 mutation in the full length CaSR, (B) the impact of E803A mutation when introduced into CaSR-B2, and (C) the functional effect of a single E803A mutation in the CaSR HHD dimer (n=3-4; error bars represent ± SEM).

Table 5. 6 Summary of Ca\textsuperscript{2+}\textsubscript{o}-sensitivity (EC\textsubscript{50}) and maximal response (E\textsubscript{max}) of E803A mutant dimers.
The EC\textsubscript{50} and E\textsubscript{max} values (±standard errors) were calculated by fitting the modified Hill equation to the integrated Ca\textsuperscript{2+}\textsubscript{i} mobilisation responses in GraphPad Prism. The Hill coefficient of E803A-B2 was fixed to 3 to enable curve fitting (significantly different a (p<0.01), b (p<0.0001) and c not significant when compared to WT).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>EC\textsubscript{50} for Ca\textsuperscript{2+}\textsubscript{o} (mM)</th>
<th>E\textsubscript{max} (IFRU)</th>
<th>Hill Coeff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT CaSR</td>
<td>3.7 ± 0.2</td>
<td>11 ± 0.2</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>E803A</td>
<td>3.0 ± 0.6\textsuperscript{c}</td>
<td>7.7 ± 0.1\textsuperscript{b}</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>E803A-B2</td>
<td>2.1 ± 0.1\textsuperscript{a}</td>
<td>7.2 ± 0.1\textsuperscript{b}</td>
<td>3</td>
</tr>
<tr>
<td>CaSR-B1 &amp; E803A-B2</td>
<td>5.3 ± 0.6\textsuperscript{c}</td>
<td>11.8 ± 0.7\textsuperscript{c}</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>E803A-B1 &amp; E803A-B2</td>
<td>4.8 ± 1.0\textsuperscript{c}</td>
<td>6.0 ± 0.7\textsuperscript{b}</td>
<td>1.3 ± 0.5</td>
</tr>
</tbody>
</table>

In contrast to F706A or L797A mutant/WT heterodimers, E803A/WT heterodimers displayed a recovery in CaSR-mediated Ca\textsuperscript{2+}\textsubscript{i} mobilisation which was comparable to WT. These findings suggest that a single E803 residue in iL-3 is required for efficient Ca\textsuperscript{2+}\textsubscript{o}-dependent Ca\textsuperscript{2+}\textsubscript{i} mobilisation. Furthermore, depending on the role of the residue in G-protein selection, binding and activation, a single HHD can be sufficient for CaSR-mediated signalling.
5.3 Discussion

5.3.1 The CaSR-GABA\_B trafficking system is suitable for studying WT-mutant heterodimers

A trafficking system was successfully designed to study CaSR heterodimers with the objectives of investigating the minimum subunit requirements for CaSR signalling. The results described in this chapter demonstrate that the system is suitable for the purpose. Firstly, introducing selected point mutations into either the VFTD or the HHD did not interfere with total or surface expression of CaSR-B1 and CaSR-B2. Secondly, the number of functional heterodimers at the cell surface was maximised and signalling responses generated by functional CaSR-B2 receptors at the cell surface were suppressed by introducing inactivating point mutations that interfere with agonist-dependent receptor activity. In addition, since CaSR-B1 was retained intracellularly when expressed alone, any response in cells co-transfected with CaSR-B1 and mutant CaSR-B2 chimeras could be attributed to the delivery of functional heterodimers to the cell surface. Consistent with this, the functional responses of co-transfected cells was different from those of cells expressing either of the constructs individually. Furthermore, introducing the same point mutation into both CaSR-B1 and CaSR-B2 recapitulated the function of the mutant homodimer in the full length CaSR backbone.

Compared to previous studies in which the function of mutant heterodimers was assessed in HEK-293 cells that co-expressed CaSR mutants with either the WTCaSR or CaSR\_B2\_X [16, 78], the system described in this thesis reduces the level of signalling arising from wild-type homodimers. A limitation of the system described here is that due to cell surface expression of CaSR-B2, it can only be used in its current form to investigate the impact of inactivating mutations. If the surface expression of CaSR-B2 could be manipulated in the same way as
CaSR-B1, it would allow for extending the system to activating and mildly-impairing mutations. Another limitation of the system is that it lacks direct evidence for heterodimerisation such as that provided by co-immunoprecipitation or FRET-based methods.

5.3.2 A single VFTD is sufficient for signalling efficacy as assessed by Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation

G143E and R185Q are recognised naturally occurring point mutations first identified in patients with FHH that are responsible for negatively modulating Ca\textsuperscript{2+}\textsubscript{o} potency [86]. Mutant homodimers have markedly impaired function in PLC and/or Ca\textsuperscript{2+}\textsubscript{i} mobilisation assays with reduced Ca\textsuperscript{2+}\textsubscript{o}-sensitivity and signalling efficacy [86].

Using the CaSR-GABA\textsubscript{B} heterodimer expression system, I found that introducing either of these mutations into a single VFTD of the CaSR dimer resulted in a reduction of Ca\textsuperscript{2+}\textsubscript{o}-sensitivity but restoration of WT levels of signalling efficacy. This demonstrates that Ca\textsuperscript{2+}\textsubscript{o}-dependent activation of a single VFTD, and the resulting closed-open dimeric VFTD conformation is sufficient to achieve control levels of CaSR signalling efficacy, but not Ca\textsuperscript{2+}\textsubscript{o}-sensitivity. The results suggest that Ca\textsuperscript{2+}\textsubscript{o}-dependent activation of both VFTDs is required for WT levels of both Ca\textsuperscript{2+}\textsubscript{o} potency, efficacy and co-operativity.

5.3.3 Two HHDs are required for CaSR G-protein coupling via F706 and L797

F706 and L797 have been shown previously to be important for Ca\textsuperscript{2+}\textsubscript{o}-induced PLC signalling [42]. F706A and L797A significantly impair IP formation in response to Ca\textsuperscript{2+}\textsubscript{o} in mutant homodimers [42]. The present study has shown that these mutants are also important.
for CaSR G-protein coupling as they impair Ca\(^{2+}\) mobilisation, ERK phosphorylation and suppression of cAMP levels.

Interestingly, the heterodimer expression system demonstrated that introducing either of these mutations in a single HHD of the CaSR dimer failed to enhance receptor signalling above the level of mutant homodimers. Thus, the mutants significantly impaired $E_{\text{max}}$ with an efficacy of 20-50% of WT (Tables 5.5 and 5.4). The results indicate that impairing G-protein coupling in one subunit impairs the efficacy of signalling in CaSR dimers. Therefore a single, functional HHD is not sufficient for WT levels of CaSR signalling and two HHDs are required.

This is consistent with studies of mGluR-5 suggesting that two functional HHDs are required for normal G-protein coupling [79]. Interestingly, this requirement does not appear to operate in GABA\(_B\) receptors [178].

### 5.3.4 A single HHD is sufficient for G-protein selectivity

E803 is important for PLC signalling and the current study has identified its role in specifying receptor-dependent activation of G\(_{q/11}\) [42]. Interestingly, E803A mutant homodimers impaired G\(_{q/11}\)-dependent PLC activation but not G\(_{i/o}\) dependent suppression of cAMP levels. To assess whether a single WT HHD is sufficient for specifying G\(_{q/11}\) activation or whether two WT HHDs are required, I introduced the E803A mutation into the CaSR-GABA\(_B\) heterodimer trafficking system. The results indicate that a single functional HHD was sufficient to select for G\(_{q/11}\) (Figure 5. 7). Indeed Ca\(^{2+}\)-induced Ca\(^{2+}\) mobilisation in mutant-WT heterodimers exhibited Ca\(^{2+}\) potency and efficacy that were very similar to
WT (Table 5. 6). On the other hand the $E_{\text{max}}$ of E803A homodimers was markedly impaired, but $\text{Ca}^{2+}$ sensitivity was similar to WT.

It is interesting that the HHD requirements for coupling to multiple G-proteins and $G_{q/11}$ selectivity on the other hand are different and are likely to be indicative of the different processes involved in G-protein coupling compared to selectivity. One possibility is that G-protein selectivity refers to recruitment and docking of G-proteins at intracellular domains of the receptor and a single, functional HHD supports this. On the other hand G-protein coupling refers to the dissociation of the heterotrimeric G-protein and its activation, for which interactions across the HHD dimer interface are required.
Chapter 6
Chapter 6  General Discussion

6.1  Molecular determinants of G-protein coupling and selectivity

It has long been known that the CaSR is a promiscuous GPCR, able to couple to several G-proteins for intracellular signalling [11]. The roles of intracellular residues and domains in G-protein coupling and signalling, however, have not been extensively studied. Two previous mutational studies have identified residues in iL-2, iL-3 and the C-tail that are important for PLC signalling and IP₁ formation [42, 46]. We sought to further evaluate the roles of these iL residues and the C-tail identified in these studies by extending the analysis to other CaSR signalling pathways including G₉/₁₁ coupled Ca²⁺ Mobilisation, ERK1/2 phosphorylation and Gᵢₒ coupled cAMP suppression.

The findings in the current study suggest that CaSR-based molecular determinants of G-protein signalling can be divided into two groups: those involved in G-protein coupling, which support G-protein activation, and those involved in G-protein selectivity, which are involved in interactions and with specific G-proteins. F706 in iL-2 and L797 in iL-3 have been tentatively identified as molecular determinants of G-protein coupling based on impaired signalling by HEK-293 cells transfected with F706A or L797A in all four assays. E803 and the C-tail, specifically residues 866-875, have been identified as molecular determinants of selective G₉/₁₁ coupling since HEK-293 cells transfected with the E803A mutant or 866X truncation mutant had markedly impaired IP₁ accumulation, Ca²⁺ mobilisation and ERK1/2 phosphorylation, but were able to suppress cAMP at levels comparable to WT.

Molecular determinants of G-protein coupling should be required for all signalling pathways downstream of the receptor. Mutating these residues impairs receptor activation irrespective
of the pathway [101, 159, 168]. In contrast, molecular determinants of G-protein selectivity allow for selective coupling to a particular G-protein or class of G-proteins [101]. Across the GPCR superfamily, various residues in iL-2 and iL-3 are known to play roles in coupling to heterotrimeric G-proteins [160]. In the case of class C GPCRs, iL-2, iL-3 and C-tail are also important for selectively coupling to G-proteins [160]. Our study suggests that this is also true for the CaSR. And like other GPCRs, CaSR/ G-protein interactions are complex being dependent on multiple intracellular sites on the receptor to activate downstream signalling pathways [168].

The exact nature of the roles of F706, L797, E803 and the C-tail residues 866-1078 play in CaSR/G-protein binding and activation are unclear. Studies suggest that molecular determinants of G-protein coupling and selectivity support direct receptor-dependent interactions with G-proteins. In the case of unligated rhodopsin, a structural study found the C-terminal of the Gα-subunit of transducin (Gt) interacts directly with iL-3 to stabilise the active form of the receptor [194]. Due to the technical difficulties associated with crystallisation of membrane proteins other methods have been used to study receptor-G-protein interactions including FRET, which has been used successfully to study G-protein interactions with α2A-adrenergic receptors [195]. In this study the Gα-subunit was labelled with the donor fluorophore, CFP, and the intracellular domain of the receptor with the acceptor fluorophore, YFP. Fluorescence microscopy was then used to monitor the dynamics of receptor G-protein coupling.

Alternatively, the iL residues and C-tail may play indirect roles in G-protein coupling by contributing to the active conformation of the receptor, or interacting with adaptor proteins that couple to G-protein signalling pathways. FRET has also been used to monitor the changes between the active and inactive conformations of class C GPCRs [93-95].
Generating such a system for the CaSR could be used to determine whether these molecular determinants are important for receptor activity.

Adaptor proteins like RGS2 and GRK2 have been shown to regulate G-protein coupling to GPCRs by interacting with intracellular domains [196, 197]. Indeed CaSR signalling is dependent on interactions with such proteins including RGS4, GRK4 and β-arrestins [89, 198]. Therefore, it would be interesting to investigate whether the residues identified here play a role in CaSR signalling by mediating interactions with recognised adaptor proteins.

The results of the current study have implicated E803 and the CaSR C-tail in coupling to Gq/11 but not Gi/o. Along with the findings of other studies these results suggest that each class of G-proteins has a unique set of molecular determinants that are required for GPCR binding [160, 166]. The CaSR determinants for Gi/o and G12/13 selectivity are yet to be identified. Future studies should investigate the roles of iL-2 and iL-3 residues in other signalling pathways. It will be interesting for example to determine whether L704A and V705A in iL-2 as well as S794A and E799A in iL-3, which have either no effect on IP accumulation or enhance it [42], might selectively impair Gi/o and/or G12/13-dependent responses.

Understanding the molecular determinants of CaSR/G-protein coupling and selectivity also has the potential to explain the molecular basis of pathological conditions. Indeed, malignancy transformation of breast epithelial cells the CaSR has been shown to switch its G-protein selectivity from Gi to Gs [117]. Although the underlying cause is unknown, mutations in the CaSR intracellular domains could be responsible for the switching in G-protein selectivity. In the case of mGluR-6, a point mutation responsible for un-coupling from Go has been associated with congenital stationary night blindness [169].
6.2 Trafficking CaSR heterodimers to the cell surface

Kniazeff et al. (2004) described the use of an ER sorting system based on the association of the GABA\textsubscript{B1} and GABA\textsubscript{B2} C-terminal tails to study behaviour of WT- mutant mGluR-5 heterodimers with a particular focus on the minimal requirements for receptor function. Several CaSR-GABA\textsubscript{B} chimeric receptors were generated in the current study to investigate the minimum requirements for \(\text{Ca}^{2+}\)\textsubscript{o} sensing and signalling using \(\text{Ca}^{2+}\)\textsubscript{i} mobilisation as the readout.

6.2.1 The CaSR C-tail regulates receptor expression and PLC signalling

The CaSR C-tail is a dynamic regulator of expression, trafficking, and signalling [20]. In order to optimise the impact of the GABA\textsubscript{B1/2} tail system, several CaSR C-tail truncations were made. Analysis of the truncation mutants supports previous studies and also highlights some interesting new findings.

As described previously, truncation after 890 or 908 increased receptor expression at the cell surface [172]. In addition I found that these receptors had enhanced \(\text{Ca}^{2+}\)\textsubscript{o}-induced \(\text{Ca}^{2+}\)\textsubscript{i} mobilisation when expressed in HEK-293 cells. Although the removal of arginine-rich intracellular retention motifs proximal to Ser908 has been reported to enhance receptor expression, no difference was observed between the cell surface expression of CaSR\textsubscript{909X} and CaSR\textsubscript{901X} in the current study. These findings suggest that the enhancement in surface expression in the case of both these mutants arises from the loss of elements distal to S908. As there are no other motifs rich in basic residues downstream of S908, enhanced receptor expression would appear to arise from the disruption of protein-protein interactions [20]. For example, the 909X truncation eliminates one or more high affintiy filamin-A binding sites [52, 112]. Disruption of interactions with other proteins that regulate receptor trafficking...
including RAMPs, E3 ubiquitin ligase and 14-3-3 may also contribute to increased surface expression [53, 85, 90].

The CaSR C-tail is required for PLC activation as truncation after residue 865 eliminated IP accumulation and/or Ca\(^{2+}\) mobilisation and the findings of the current study support this conclusion (see Figure 3. 4, Figure 3. 5) [45-47].

In the current study the truncation mutant 876X exhibited Ca\(^{2+}\)-induce Ca\(^{2+}\) responses that took the form of sustained increases rather than the repetitive low frequency Ca\(^{2+}\) oscillations consistent with the idea that the primary PKC phosphorylation site, T888, is required for the normal control of Ca\(^{2+}\) oscillations although other sites may contribute [49, 105, 182]. Analysis of the Ca\(^{2+}\) mobilisation responses of HEK-293 cells transfected with 891X suggested that a minimum number of residues after T888 support efficient PKC phosphorylation. Thus, Ca\(^{2+}\)-induced Ca\(^{2+}\) oscillations were not observed in HEK-293 cells transfected with 891X (Figure 4. 5). Interestingly, Ca\(^{2+}\)-induced Ca\(^{2+}\) oscillations were restored in the chimeric construct CaSR\(_{890}\)-B1 in which the GABA\(_B1\) C-tail was appended after R890. To determine the minimum requirements for Ca\(^{2+}\) oscillations, further studies with truncated mutants possessing progressively longer C-tails after S890 need to be performed.

### 6.2.2 Manipulation of CaSR C-tail regulates cell surface expression

As observed in the current study and reported previously [45-47, 172], CaSR C-tail truncation can be used to manipulate the level of receptor surface expression dependent on the site of truncation. This study has shown that the addition of motifs including the GABA\(_{B1}\)
C-tail (residues 854-960) and the peptide, KKTN, can also be used to manipulate CaSR cell surface expression.

The addition of two different intracellular retention motifs impaired cell surface expression of CaSR909X. Appending the GABA_B1 C-tail to the C-terminus of 909X impaired expression by around 50% of WT. Alternatively, appending a truncated form of the GABA_B2 C-tail followed by addition of the ER retention motif, KKTN, also markedly suppressed cell surface expression by around 75%. Interestingly, despite the marked impairment of surface expression, receptor function, as reported by Ca^{2+} mobilisation, was relatively preserved (Figure 4.9), although a small reduction in the E_{max} of CaSR-B2_{KKTNX} was observed compared to CaSR-B2.

In HEK-293 cells expressing CaSR_{875-B1}, where the GABA_B1 C-tail was appended to CaSR_{876X}, like CaSR_{908-B1}, had surface expression reduced to around 50%, but the function was abolished. These findings suggest that the functional significance of receptor density is also dependent on the amount of CaSR C-tail a receptor possesses. The exact nature of this relationship is unknown though it would seem findings of the current study with CaSR_{909X}, CaSR_{876X} and their GABA_B1 chimeras, suggest that the greater the amount of the CaSR C-tail a receptor possesses, the smaller the amount of receptors needed at the cell surface for CaSR function.

### 6.2.3 CaSR heterodimer trafficking

By replacing the CaSR C-tail with those of either GABA_B1 or GABA_B2, I have developed a system to study WT-mutant CaSR heterodimers.
The C-tails of the \textit{GABA}_{B1} and \textit{GABA}_{B2} receptors contain motifs responsible for intracellular retention and forward trafficking, respectively [177, 181]. Kniazeff \textit{et al.} showed that replacing residues 845-1171 of the mGluR-5 C-tail with those of the \textit{GABA}_{B1} (residues 854-960) and \textit{GABA}_{B2} (760-941) permitted the development of an ER sorting system suitable for the delivery of mutant heterodimers to the cell surface [79, 174]. They proposed this method could also be applied to other class C GPCRs. The current study confirms this by showing that the addition of the \textit{GABA}_{B1} C-tail to various CaSR constructs (CaSR-B1) reduces the level of surface expression without affecting total expression. Thus, the results indicate that the appended \textit{GABA}_{B1} tails confer intracellular retention consistent with its role in the formation of \textit{GABA}_{B} heterodimers [177]. The surface expression of CaSR-B1 was enhanced by co-expression with a CaSR chimera possessing the \textit{GABA}_{B2} C-tail (CaSR-B2), presumably through heterodimer formation (Figure 4.11).

Although CaSR-B2 homodimers were expressed efficiently at the cell surface and were functional (Figure 4.8, Figure 4.9), their function was impaired by the introduction of specific point mutations, as shown previously [79].

We also attempted to reduce CaSR-B2 cell surface expression by introducing the KKTN intracellular retention motif after the coiled-coil domain in the \textit{GABA}_{B2} tail (CaSR-B2_{KKTNX}), as has been shown previously [99]. Although this construct exhibited impaired surface expression as noted above, receptor function, as reported by \( \text{Ca}^{2+}_o \)-induced \( \text{Ca}^{2+}_i \) mobilisation was surprisingly preserved with no apparent change in the EC$_{50}$ for \( \text{Ca}^{2+}_o \) and a reduction in E$_{max}$ of only around 50%. This finding indicates that C-tail motif(s) are present proximal to S908, which support receptor function at the surface despite significantly impaired surface expression. Further work needs to be carried out to identify the motif(s).
In addition, the surface expression of CaSR-B2\textsubscript{KKTN}X was not enhanced when it was co-expressed with various CaSR-B1 chimeras. Future work should consider optimising CaSR-B2\textsubscript{KKTN}X construct by further truncating the CaSR C-tail.

I observed an increase in the surface expression of CaSR-B1 and a small reduction in the surface expression of CaSR-B2 when they were co-expressed. In addition the functional analysis demonstrated that cells co-expressing CaSR-B1 and mutated CaSR-B2 variants exhibit functional complementation compared to HEK-293 cells transfected with the individual constructs. Taken together these results strongly support the formation and trafficking of heterodimers composed of CaSR-B1 and CaSR-B2 to the cell surface. However, studies are required to establish the existence of direct interactions between the receptor subunits. In one approach two different antibody-binding epitopes could be inserted into each of the two receptors and direct contact confirmed by co-immunoprecipitation or by the use of FRET acceptor- and donor-labelled antibodies [78, 79, 99].

### 6.3 Minimum CaSR dimer requirements

#### 6.3.1 Requirements for nutrient-sensing

Using the CaSR heterodimer expression system, I found that a single responsive VFTD was sufficient for CaSR activation. This was demonstrated by the introduction of the inactivating point mutations, G143E or R185Q into the CaSR-B2 construct [78, 86]. In cells expressing WT-mutant heterodimers possessing either of these mutations, $E_{\text{max}}$ was comparable to WT, however similar losses in Ca$^{2+}$-sensitivity were observed (Figure 5. 3-4, Table 5. 2-3). This suggests that two functional VFTDs are required for WT Ca$^{2+}$-sensitivity, but that only one is required for WT signalling efficacy.
These results are similar to those reported for mGluR-5 GABA$_{B1}$/GABA$_{B2}$ heterodimers in which the binding of quisqualate in a single VFTD was sufficient for partial activity, but the binding of quisqualate in both VFTDs was necessary for full receptor activation [79]. Kniazeff et al. suggested that the closed/open VFTD conformation in the mGluR-5 dimer was sufficient for activation, but that closure of both VFTDs was required for full activity.

I expect that the CaSR heterodimer trafficking system developed in the current study may also be applied to study the requirements of other CaSR ligands including the PAMs L-Phe and cinacalcet, as well as NAMS such as NPS 2143. Candidate mutations include the T145A/S170T double mutation and F684A which are insensitive to L-Phe and phenylalkylamines, respectively [28, 41]. As in the case of mGluR-5, it can be hypothesised that the binding of a PAM in a single subunit would be sufficient to enhance the potency of the agonist for the CaSR as well [183].

### 6.3.2 Requirements for CaSR signalling

The impaired signalling observed with WT-VFTD mutant heterodimers may have arisen from a single HHD. The results show that mutations that impair Ca$^{2+}$o-induced Ca$^{2+}$i mobilisation in homodimers, have different effects in heterodimers depending on the mutation. If a single HHD possessed either the F706A or L797A mutation in the heterodimer, no functional response to Ca$^{2+}$o was observed up to a concentration of 30 mM. If E803A was present in a single HHD however, the receptor response was comparable to the WT. In light of the roles established for F706 and L797 to various G-protein dependent pathways, and E803 in selecting for G$_{q/11}$-dependent pathways, it is tempting to conclude that two HHDs are required for efficient G-protein activation, but that only a single HHD is required for G$_{q/11}$ selectivity. This finding is in contrast with the results reported for quisqualate activated
mGluR-5 in which a single functional HHD resulted in partial activity, suggesting that the HHD requirements for signalling vary either between members of class C GPCRs or in response to distinct ligands [79]. Further work is required to establish the molecular determinants of G-protein coupling and selectivity.

6.3.3  Further applications of the heterodimer trafficking system

I have developed a heterodimeric CaSR trafficking system and have used it to study the subunit requirements of Ca$^{2+}$-induced mobilisation. This system also holds great potential for investigating the subunit requirements of other signalling pathways including $G_{i/o}$ coupled cAMP inhibition, ERK1/2 phosphorylation and IP accumulation.
6.4 Main Conclusions

1. Assessing the role of CaSR domains and residues critical for PI-PLC signalling in other CaSR-mediated signalling pathways has demonstrated that the CaSR exhibits distinct molecular determinants for G-protein coupling and selectivity. F706 in iL-2 and L797 in iL-3 have been identified as key determinants of G-protein coupling in the activation of pathways downstream of $G_{q/11}$ and $G_{i/o}$. E803 and CaSR C-tail residues 866-875 have been identified as molecular determinants of $G_{q/11}$ selectivity as they are required for IP$_1$ accumulation, Ca$^{2+}$i mobilisation and ERK1/2 phosphorylation, but not suppression of cAMP levels.

2. A heterodimer trafficking system composed of CaSR-GABA$_{B_1}$ and CaSR-GABA$_{B_2}$ chimeras suitable for studying WT-mutant CaSR heterodimers based on a previously described mGluR-5 GABA$_B$ system [79] was successfully developed. Appending the GABA$_{B_1}$ C-tail (residues 854-960) after S875 in the CaSR C-tail was found to suppress receptor surface expression resulting in the loss of Ca$^{2+}$o-induced Ca$^{2+}$i mobilisation.

Co-expression of a CaSR-B2 construct, in which residues 760-942 of the GABA$_{B_2}$ C-tail were appended after S908 in the CaSR C-tail, successfully trafficked CaSR-B1 to the cell surface in an interaction that was dependent on the GABA$_{B_1}$ and GABA$_{B_2}$ C-tails.

3. The CaSR-GABA$_B$ heterodimer trafficking system was used to study the Ca$^{2+}$o-induced Ca$^{2+}$i mobilisation of mutant heterodimers possessing an inactivating
mutation in a single subunit, allowing for the study of minimum subunit requirements for CaSR signalling.

4. The application of the system has identified the subunit requirements of CaSR function as reported by $\text{Ca}^{2+}\text{o}$-stimulated $\text{Ca}^{2+}\text{i}$ mobilisation, as follows:

   - a single functional VFTD is required for attaining maximal efficacy of $\text{Ca}^{2+}\text{o}$-induced $\text{Ca}^{2+}\text{i}$ mobilisation,
   - two functional VFTDs are required to support wild-type $\text{Ca}^{2+}\text{o}$-sensitivity and efficacy,
   - two functional HHDs are required for G-protein dependent coupling to pluripotent pathways, and
   - a single functional HHD is required for the selection of $\text{G}_q/11$ and perhaps other G-proteins.
References
Chapter 7 References

19. Ray, K., et al., Identification of the Sites of N-Linked Glycosylation on the Human Calcium Receptor and Assessment of Their Role in Cell Surface Expression and


Godwin, S.L. and S.P. Soltoff, Calcium-sensing receptor-mediated activation of phospholipase C-γ1 is downstream of phospholipase C-β and protein kinase C in MC3T3-E1 osteoblasts. Bone, 2002. 30(4): p. 559-566.


Fromigué, O., et al., Calcium-sensing receptor-dependent and independent mechanisms are involved in the increased osteoblast replication and survival by strontium ranelate. Bone, 2009. 44, Supplement 2(0): p. S313-S314.


Appendices
Appendix I

Table 1: PCR Primers for generating CaSR-GABA<sub>B1</sub> chimeras
Restriction sites are shown in italics, CaR binding regions are underlined. Each PCR reaction was performed with 5’ Kpn I primer (forward) and one of the three reverse primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ Kpn I primer (forward)</td>
<td>CAG TAT GGT ACC ATG GCA TTT TAT AGC TGC TGC T</td>
</tr>
<tr>
<td>3’ Bam HI CaR&lt;sub&gt;890&lt;/sub&gt; (reverse)</td>
<td>TAGACT GGA TCC AGC GTG AGC TGC GGT GCT GCA A</td>
</tr>
<tr>
<td>3’ Bam HI CaR&lt;sub&gt;875&lt;/sub&gt; (reverse)</td>
<td>AGACT GGA TCC GCT GCA ACG CAC CTC CTC GAT GGT</td>
</tr>
<tr>
<td>3’ Bam HI CaR&lt;sub&gt;865&lt;/sub&gt; (reverse)</td>
<td>TAGACT GGA TCC GGA TGG CTT GAA GAG AAT GAT</td>
</tr>
</tbody>
</table>

Table 2: PCR Primers for truncating CaSR C-tail.
Restriction sites are shown in italics, CaSR binding regions are underlined and introduced stop codons are highlighted in red. PCR reactions for each of the four truncations were performed using the 5’ Kpn I primer (forward) in conjunction with one of the four reverse primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ Kpn I primer (forward)</td>
<td>CAG TAT GGT ACC ATG GCA TTT TAT AGC TGC TGC T</td>
</tr>
<tr>
<td>3’ Xba I CaR&lt;sub&gt;969&lt;/sub&gt; X (reverse)</td>
<td>GACT TCT AGA TTA GGA TCC CGT GGA GCC TCC AA</td>
</tr>
<tr>
<td>3’ Xba I CaR&lt;sub&gt;801&lt;/sub&gt; X (reverse)</td>
<td>GACT TCT AGA TTA GCG CAG CGT GGC CCG GGC A</td>
</tr>
<tr>
<td>3’ Xba I CaR&lt;sub&gt;876&lt;/sub&gt; X (reverse)</td>
<td>AGACT TCT AGA TTA GCT GCA ACG CAC CTC CTC GAT GGT</td>
</tr>
<tr>
<td>3’ Xba I CaR&lt;sub&gt;866&lt;/sub&gt; X (reverse)</td>
<td>TAGACT TCT AGA TTA GGA TGG CTT GAA GAG AAT GAT</td>
</tr>
</tbody>
</table>
Appendix II

Table 3: PCR primers used to generate CaSR-B2(KKTNX).
Restriction sites are shown in italics, CaR binding regions are underlined, GABA\textsubscript{B2} tail binding regions are shown in bold and GABA\textsubscript{B2} tail truncation and retention motif are shown in red

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ Kpn I primer (forward)</td>
<td>CAG TAT \textit{GGT ACC} ATG GCA TTT TAT AGC TGC TGC T</td>
</tr>
<tr>
<td>3’ Xba I \textsubscript{B2} GABA\textsubscript{B2} (KKTNX) (reverse)</td>
<td>G TAT \textit{TCT AGA} \textbf{TTA ATT CGT CTT CTT T} \texttt{TTC TGG TGT GTC} CTG CAG CTG C</td>
</tr>
</tbody>
</table>

Table 4: PCR primers used to introduce point mutation in the GABA\textsubscript{B1} C-tail by site-directed mutagenesis.
The mutation changed the ER retention motif from RSRR to ASAR. The nucleotide mutation is underlined and shown in red in the sequence.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
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</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>CAG TCT CGG CAG CAA CTC \underline{GCC} TCA \underline{GCC} CGC CA</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>TGT TGG GGG GTG GCG \underline{GC} TGA \underline{GC} GAG TTG CT</td>
</tr>
</tbody>
</table>
**Appendix III**

Table 5: PCR primers used to introduce point mutations in the CaSR cDNA by site-directed mutagenesis.

For each amino acid mutation, the codon encoding the mutation is underlined.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G143E</strong></td>
<td>Forward</td>
<td>GATTGCTGTGGTGGAAGCAACTGGCTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTCCGGAGCCAGTGTGCTTTCCACCACAGC</td>
</tr>
<tr>
<td><strong>R185Q</strong></td>
<td>Forward</td>
<td>CAAGTCTTTTCTCCAAACCATCCCCCAATGATG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CATCATTGGGGATGGTTGGAGGAAGACTTG</td>
</tr>
<tr>
<td><strong>F706A</strong></td>
<td>Forward</td>
<td>CCAACCGTGCTCCTGGTGGCTGAGGCAAGAT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGGGGATCTTGGCCTCAGCCACCAGGAGGACA</td>
</tr>
<tr>
<td><strong>L797A</strong></td>
<td>Forward</td>
<td>TTCAAGTCGCCGGAAGGGCCGGAGAAAATCAATGAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAGTTCTCCGGGCCCTCCGGGACTGAAAGGCAA</td>
</tr>
<tr>
<td><strong>E803A</strong></td>
<td>Forward</td>
<td>GCCGGAAGAATTTCAATGCAGCCAAGTTCCATCACCTTTCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTGATGAATTTGGCTGCAATTGAAAGTTCTCCGGCAGCTTC</td>
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</table>