Characterisation of Calcium-Sensing Receptor
Extracellular pH Sensitivity and Intracellular
Signal Integration

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ABBREVIATIONS

i. 1,25-dihydroxyvitamin D₃
ii. 1,9dIFSK – 1,9 dideoxyforskolin
iii. 6-Bnz-cAMP - N6- Benzoyladenosine- 3', 5'- cyclic monophosphate
iv. 8-pCPT-2'-O-Me-cAMP - pCPT-cAMP
v. AA – Arachidonic acid
vi. AACOCF₃ - Arachidonyl trifluoromethyl ketone
vii. AC – Adenylate cyclase
viii. ACTH - Adrenocorticotrophic hormone
ix. ADH – Autosomal dominant hypocalcaemia
x. ADIS – Agonist-dependent insertional signalling
xi. AE2 – Anion exchanger 2
xii. AGAs – Aminoglycosides antibiotics
xiii. APs – Adapter proteins
xiv. APS – Ammonium persulphate
xv. AQP2 – Aquaporin 2
xvi. BCECF - 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein
xvii. Bovine parathyroid gland - bPTG
xviii. BLAST – Basic Local Alignment Search Tool
xix. Ca²⁺ – Intracellular calcium
xx. Ca²⁺₀ – Extracellular calcium
xxi. cAMP – Cyclic adenosinemonophosphate
xxii. CaR – Calcium sensing receptor
xxiii. CCD – Cortical collecting duct
xxiv. CCV – Clathrin coated vesicle
xxv. CKD – Chronic kidney disease
xxvi. COBALT - Constraint-based Multiple Protein Alignment Tool
xxvii. cTAL – cortical thick ascending limb
xxviii. DAG – Diacylglycerol
xxix. DCT – Distal convoluted tubule
xxx. DEPC - Diethylpyrocarbonate
xxx. DMEM- Dulbecco’s minimum Eagle’s medium
xxxii. DNA – Deoxyribonucleic acid
xxxiii. ECD – Extracellular domain
xxxiv. EDTA - Ethylenediaminetetraacetic acid
xxxv. EGTA - Ethylene glycol tetraacetic acid
xxxvi. EPAC - Exchange protein directly activated by cAMP
xxxvii. ER – Endoplasmic reticulum
xxxviii. ERK – Extracellular signal-regulated kinase
xxxix. FBS – Foetal Bovine Serum
xl. FHH – Familial Hypercalcaemia Hypocalciuria
xli. FRET – Fluorescence resonance energy transfer
xlii. FSK - Forskolin
xliii. GABAbR – γ aminobutyric acid b receptor
xliv. GI – Gastrointestinal
xl. GPCR – G protein-coupled receptor
xli. GPR4 – G-protein-couple receptor 4
xlii. HCN2 - Hyperpolarisation-activated cyclic nucleotide-gated ion channel 2
xliv. HEK – Human embryonic kidney
xlv. HRP- Horseradish peroxidase
l. IBMX - 3-isobutyl-1-methylxanthine
li. ICD – Intracellular domain
lii. IKCa – Intermediate conductance calcium activated potassium channel
liii. IMCD – Inner medullary collecting duct
liv. IP- Inositol phosphate
lv. IP3 – Inositol trisphosphate
lvi. JNK- cJun N-terminal Kinase
lvii. KO – Knock-out
lviii. MCD – Medullary collecting duct
lix. MDCK cells – Madin-Darby canine kidney cells
lx. MEK – Mitogen-activated protein kinase
lxi. mGluR – Metabotropic glutamate receptor
lxii. NCX – Sodium-calcium exchanger
lxiii. NEM - N-Ethyl maleimide
lxiv. NKCC2 – Sodium Potassium Chloride cotransporter isoform 2
lxv. NSHPT – Neonatal severe hyperparathyroidism
lxvi. OGR1 - Ovarian cancer G protein-coupled receptor 1
lxvii. OK cells – Opossum Kidney cells
lxviii. PA – Phosphatidic Acid
lxix. PBS – Phosphate-buffered saline
lxx. PCT – Proximal convoluted tubule
lxxi. PIP2- Phosphoinositol 4,5-bisphosphate
lxxii. PKA – Protein Kinase A
lxxiii. PKB – Protein Kinase B
lxxiv. PKC – Protein Kinase C
lxxv. PLA_2 – Phospholipase A_2
lxxvi. PLC – Phospholipase C
lxxvii. PMCA – Plasma membrane calcium ATPase
lxxviii. PP- Protein phosphatase
lxxix. PP2A- Protein phosphatase 2A
lxxx. PT – Parathyroid
lxxxi. PTH – Parathyroid hormone
lxxxii. PTHrP – Parathyroid hormone-related protein
lxxxiii. PTx- Pertussis Toxin
lxxxiv. RIPA buffer – Radioactive immunoprecipitation assay buffer
lxxxv. ROMK channel - Renal outer medullary potassium channel
lxxxvi. rtPCR- Reverse transcriptase PCR
lxxxvii. SDS- Sodium dodecyl sulphate
lxxxviii. SFO – Subfornical organ
lxxxix. SHPT – Secondary hyperparathyroidism
  xc. siRNA – Small interfering ribonucleic acid
  xci. SK4 – Calcium activated potassium channel 4
  xcii. SKCa – Small conductance calcium-activated potassium channel
  xciii. SRE – Serum response element
  xcv. TMD – Transmembrane domain
  xcv. TPA/PMA – Phorbol ester
  xcvi. TRPC1 - Transient receptor potential channel 1
  xcvii. TRPV5 - Transient receptor potential cation channel subfamily V member 5
  xcviii. VFD – Venus flytrap domain
  xcix. VIP- Vasoactive intestinal peptide
    c. VSMCs – Vascular smooth muscle cells
ABSTRACT
Parathyroid hormone (PTH) secretion maintains free-ionised extracellular calcium (Ca\textsuperscript{2+}) homeostasis under the control of the calcium-sensing receptor (CaR). In humans and dogs, blood acidosis and alkalosis is associated with increased or suppressed PTH secretion respectively. Furthermore, large (1.0 pH unit) changes in extracellular pH (pH\textsubscript{o}) alter Ca\textsuperscript{2+} sensitivity of the CaR in CaR-transfected HEK-293 cells (CaR-HEK). Indeed, it has been found in this laboratory that even pathophysiological acidosis (pH 7.2) renders CaR less sensitive to Ca\textsuperscript{2+} while pathophysiological alkalosis (pH 7.6) increases its Ca\textsuperscript{2+} sensitivity, both in CaR-HEK and parathyroid cells. If true \textit{in vivo}, then CaR's pH\textsubscript{o} sensitivity might represent a mechanistic link between metabolic acidosis and hyperparathyroidism in ageing and renal disease. However, in acidosis one might speculate that the additional H\textsuperscript{+} could displace Ca\textsuperscript{2+} bound to plasma albumin, thus increasing free-Ca\textsuperscript{2+} concentration and so compensating for the decreased CaR responsiveness. Therefore, I first demonstrated that a physiologically-relevant concentration of albumin (5% w/v) failed to overcome the inhibitory effect of pH 7.2 or stimulatory effect of pH 7.6 on CaR-induced intracellular Ca\textsuperscript{2+} (Ca\textsuperscript{2+}) mobilisation.

Determining the molecular basis of CaR pH\textsubscript{o} sensitivity would help explain cationic activation of CaR and permit the generation of experimental CaR models that specifically lack pH\textsubscript{o} sensitivity. With extracellular histidine and free cysteine residues the most likely candidates for pH\textsubscript{o} sensing (given their sidechains' pK values), all 17 such CaR residues were mutated to non-ionisable residues. However, none of the resulting CaR mutants exhibited significantly decreased CaR pH\textsubscript{o} sensitivity. Even co-mutation of the two residues whose individual mutation appeared to elicit modest reductions (CaR\textsuperscript{H429V} and CaR\textsuperscript{H495V}) failed to exhibit any change in CaR pH\textsubscript{o} sensitivity. I conclude therefore, that neither extracellular histidine nor free cysteine residues account for CaR pH\textsubscript{o} sensitivity.

Next, it is known that cytosolic cAMP drives PTH secretion \textit{in vivo} and that cAMP potentiates Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation in CaR-HEK cells. Given the physiological importance of tightly controlled PTH secretion and Ca\textsuperscript{2+}\textsubscript{o} homeostasis, here I investigated the influence of cAMP on CaR signalling in CaR-HEK cells. Agents that increase cytosolic cAMP levels such as forskolin and isoproterenol potentiated Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation and lowered the Ca\textsuperscript{2+}\textsubscript{o} threshold for Ca\textsuperscript{2+}\textsubscript{i} mobilisation. Indeed, forskolin lowered the EC\textsubscript{50} for Ca\textsuperscript{2+}\textsubscript{o} on CaR (2.3 ± 0.1 vs. 3.0 ± 0.1 mM control, P<0.001). Forskolin also potentiated CaR-induced ERK phosphorylation; however protein kinase A activation appeared uninvolved in any of these effects. Pertussis toxin, used to block CaR-induced suppression of cAMP accumulation, also lowered the Ca\textsuperscript{2+}\textsubscript{o} threshold for Ca\textsuperscript{2+}\textsubscript{i} mobilisation though appeared to do so by increasing efficacy (E\textsubscript{max}). Furthermore, mutation of the CaR's two putative PKA consensus sequences (CaR\textsuperscript{S899} and CaR\textsuperscript{S900}) to a non-phosphorylatable residue (alanine) failed to alter the potency of Ca\textsuperscript{2+}\textsubscript{o} for CaR or attenuate the forskolin response. In contrast, phosphomimetic mutation of CaR\textsuperscript{S899} (to aspartate) did increase CaR sensitivity to Ca\textsuperscript{2+}\textsubscript{o}. Together this suggests that PKA-mediated CaR\textsuperscript{S899} phosphorylation could potentiate CaR activity but that this does not occur following Ca\textsuperscript{2+}\textsubscript{o} treatment in CaR-HEK cells. Together, these data show that cAMP regulates the Ca\textsuperscript{2+}\textsubscript{o} threshold for Ca\textsuperscript{2+}\textsubscript{i} mobilisation, thus helping to explain differential efficacy between CaR downstream signals. If true \textit{in vivo}, this could help explain how multiple physiological signal inputs may be integrated in parathyroid cells.
DECLARATION

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Chapter 1

Introduction
1.1 Extracellular calcium homeostasis

The precise maintenance of extracellular calcium homeostasis is vital for human life given the wide array of fundamental physiological processes for which calcium is essential. Calcium is the primary inorganic cation in bone; it is involved in muscle contraction, neurotransmitter and hormone release, intracellular communication, DNA synthesis and enzyme activity (Brown & MacLeod, 2001). Deviation from normal calcium concentrations can cause hypercalcaemic arrhythmias and hypocalcaemic tetany and is linked to a number of chronic diseases including osteoporosis, kidney stone formation (nephrolithiasis) and vascular calcification, and, even to obesity, heart disease and hypertension (Hendy et al, 2009). As terrestrial mammals we rely on our diet as our fundamental source of calcium and upon the secretion of parathyroid hormone (PTH) as our primary mechanism for the maintenance of an appropriate plasma calcium concentration.

1.1.1 Discovery of the parathyroid gland and its role in calcium homeostasis

The parathyroid glands were first discovered by medical student Ivar Sandström in 1880. However their existence was largely dismissed and little research was conducted to determine their function. Interest returned in the 1900s when MacCallum and Voegtlin linked the removal of the parathyroid glands to tetany and disturbances in calcium homeostasis (MacCallum & Voegtlin, 1976). However, their findings were met with scepticism, as one scientist of the time remarked:

“For this scepticism there are perhaps two reasons: one, the difficulty of apprehending the fact of such small and insignificant bodies being charged with such great importance to the health of the human body...” (Brown, 1911).

The two main trains of thought were that the glands were either embryonic thyroid glands based on anatomical evidence, or, that they had a separate function concerning calcium and tetany, based on physiological and experimental evidence. Physicians began administering patients whole bovine parathyroid glands orally, or in an emulsion to cure tetany (Berkeley & Beebe, 1909; Halsted & Evans, 1907; MacCallum & Voegtlin, 1976). Another technique used was to place bovine, simian or human parathyroid glands under the skin (Mayo, 1909). In 1906, Silas Beebe described a “nucleo-protid” extract from the parathyroid glands that alleviated the symptoms on tetany and could be used multiple times (Beebe, 1906). His preparation is cited a number of times by different physicians who successfully
treated patients for tetany following removal of the parathyroids (Berkeley & Beebe, 1909). Following on from this in the 1920s, James Collip and others developed a stable, clinically viable parathyroid hormone preparation (Collip, 1925). His standardised solution made it possible for physicians to treat patients who had undergone parathyroidectomy or suffered from hypoparathyroidism.

Work continued examining the relationship between PTH, vitamin D, phosphorus and calcium homeostasis. It was understood that low calcium concentrations led to the secretion of PTH, however the exact mechanism was unclear (McLean, 1957). Development of radioimmunoassays and animal models with greater sensitivity allowed the discovery of the inverse relationship between free-ionised calcium concentration and PTH secretion (Care et al, 1966; Sherwood et al, 1968; Sherwood et al, 1966).

By the 1970s and 80s it had been clearly established that the parathyroid glands play the central role in extracellular calcium homeostasis but it remained unclear how extracellular calcium fed back on the gland to suppress PTH secretion. It was noted that agonists of endogenously expressed Gαs-protein linked receptors, which increased cAMP, drove PTH secretion (Brown et al, 1977a; Brown et al, 1977b; Gardner et al, 1978). Although there was some evidence that a GPCR contributed to PTH secretion, the primary hypothesis was that a calcium channel was involved (Fitzpatrick et al, 1986b).

1.1.2 Discovery of the calcium-sensing receptor and its role in calcium homeostasis

The existence of a calcium-sensing receptor was first postulated by Lopez-Barneo and Armstrong in the 1983 (López-Barneo & Armstrong, 1983). This proposal was corroborated by Brown and Nemeth; collectively, their data showed that acute phorbol ester (PMA), an activator of protein kinase C (PKC), stimulated PTH secretion independently of cAMP and extracellular calcium (Ca\(^{2+}\))(Brown et al, 1987; Brown et al, 1984; Nemeth & Scarpa, 1986). Therefore it was hypothesised that activation of PKC causes phosphorylation of an unknown factor resulting in PTH secretion. Increasing Ca\(^{2+}\) also increased inositol trisphosphate (IP\(_3\)) and diacylglycerol (DAG) levels and indicated the involvement of phospholipase C (PLC), almost certainly activated by a G-protein coupled receptor (GPCR). The development of intracellular calcium (Ca\(^{2+}\)) indicators with greater spatial and temporal resolution enabled the observation that increases in Ca\(^{2+}\), were not
monophasic but transient followed by a sustained response and is indicative of intracellular calcium release rather than extracellular calcium influx (Nemeth & Carafoli, 1990; Nemeth & Scarpa, 1987; Nemeth et al, 1986). It was also observed that other divalent cations produced the same effect independently of calcium. These results indicated a cell-surface receptor capable of ‘sensing’ extracellular divalent cations (Brown, 1991). However, their conclusions were widely discredited and it was not until 1993 when the identity of the calcium-sensing receptor (CaR) was discovered by Ed Brown and co-workers, in bovine parathyroid gland, that their work was endorsed (Brown et al, 1993). The expression and function of the CaR has since been extensively researched and it is now known to be not only involved in calcium homeostasis but a wide range of other cell functions (see section 1.3).

Fractionated bovine parathyroid mRNA isolated by Brown et al produced a 5.3 kilobase (kb) complementary DNA (cDNA) clone; capable of producing Gd$^{3+}$-activated Cl$^{-}$ currents in *Xenopus laevis* oocytes (Brown et al, 1993). Currents were produced by activation of PLC, which initiated Ca$^{2+}$ mobilisation, in-turn stimulating Ca$^{2+}$-activated Cl$^{-}$ channels. Sequencing the 5.3 kb cDNA revealed a 1085 amino acid membrane protein, coded in a 3255 bp open reading frame. Two years later the human CaR was cloned from parathyroid gland from a patient with primary hyperparathyroidism (Garrett et al, 1995). Human CaR is 1078 amino acids in length and shares 93% homology with bovine CaR. The majority of non-conserved amino acids are in the intracellular domain, inferring a difference in intracellular signalling. Bovine CaR has 9 N-linked glycosylation and 4 PKC sites. Human CaR was predicted to have 11 glycosylation sites, 5 PKC consensus sites `sites and 2 protein kinase A (PKA) phosphorylation sites. The CaR has since been cloned in a number of other animals including chicken, rat, rabbit, dog and fish species (Butters et al, 1997; Diaz et al, 1997; Loretz et al, 2004; Naito et al, 1998; Nearing et al, 2002; Riccardi et al, 1995). The human CaR gene region was isolated to chromosome 3q13.3-21 using fluorescence in situ hybridisation (Janicic et al, 1995). The gene did not appear to hybridise anywhere else inferring that it is a single copy gene. Human CaR gene transcription appears to be under the control of two different promoters; the first upstream of exon 1A contains TATA and CAAT boxes whereas the second is between exons 1A and 1B and is GC-rich. Having two separate promoters implies that the CaR gene could have tissue-specific transcription (Chikatsu et al, 2000).
1.2 Calcium-sensing receptor structure

The CaR is a member of family C of the GPCR superfamily of receptors, along with the γ-aminobutyric acid type B (GABAb) receptor, metabotropic glutamate (mGluR) receptors, the taste receptors, vomeronasal and orphan receptors (Brauner-Osborne et al, 1999; Brauner-Osborne et al, 2007; Hermans & Challiss, 2001; Romano et al, 1996; Wise et al, 1999). They exhibit classic GPCR structure with seven transmembrane domains (TMD), an internal C-terminus which binds G proteins but their most distinctive feature is a very large extracellular N-terminus domain (Brown et al, 1993).

1.2.1 Extracellular domain

The large human CaR extracellular domain (ECD) is 612 amino acids long and contains regions conserved between CaR and mGluRs (Garrett et al, 1995). The ECD contains the ligand-binding region, which is reminiscent of gram-negative bacteria periplasmic binding proteins (PBPs), and forms a bilobed Venus flytrap domain (VFD) (Felder et al, 1999; O’Hara et al, 1993; Ray et al, 1999; Reyes-Cruz et al, 2001; Silve et al, 2005). The ECD also has a cysteine-rich region containing 20 conserved cysteines in the ECD and intracellular loops of the TMD (Hu et al, 2001; Ray et al, 1999).

1.2.2 Venus flytrap and calcium binding domains

Sequence alignment analysis showed that the mGluRs’ ECDs had considerable homology to the PBPs (O’Hara et al, 1993). Aligning the CaR and mGluR1 showed substantial homology between amino acids 36 to 513 in the ECD and the CaR was modelled on the PBPs (Ray et al, 1999). The VFD extends between amino acids 20 and 536 and contains the Ca\(^{2+}\) binding regions. The VFD contains two globular lobes containing β-sheets, connected by three stands (Ray et al, 1999). Ligand binding causes the lobes to twist and close, stabilising the confirmation of the protein (Felder et al, 1999). Nine highly conserved cysteines are present in the N-terminal domain, between the VFT and transmembrane domain (Bai, 2004).

One study showed that mutation of 14 of the 19 conserved cysteine residues in the ECD caused ER retention of the receptor (Fan et al, 1998). Mutation of the remaining 5 did not perturb the ability of the receptor to sense calcium (Fan et al,
Determining the Ca$^{2+}$-binding region has proven very complex due to the difficulty of crystallising the structure. Putative models of the calcium binding regions are based on computer algorithms employing the X-ray structure of mGluR1. The CaR has a Hill coefficient of between 3 and 5, which implies cooperativity of binding of Ca$^{2+}$ ions. Silve and Huang both concluded independently that Ser-147, Ser-170, Asp-190, Gln-193, Tyr-218, Phe-270, Ser-296 and Glu-297 formed a Ca$^{2+}$ binding pocket (Huang et al., 2007b; Silve et al., 2005). Mutating each of these sites to either Ala or Lys caused a significant decrease in receptor maximal activity versus WT (Silve et al., 2005; Zhang et al., 2002b). In addition to this binding site Huang et al. also reported two further putative Ca$^{2+}$ binding sites; Glu-378, Glu-379, Thr-396, Asp-398 and Glu-399 located in lobe 1 of the VFD and site three, Glu-224, Glu-228, Glu-229, Glu-231 and Glu-232 located in lobe 2 of the VFD (Huang et al., 2007b). Natural occurring mutations of two of these residues has been shown to cause disease; E228Q causes ADH (Conley et al., 2000) and Y218S causes FHH (Pearce et al., 1995).

1.2.3 Glycosylation and the ECD

Glycosylation is the post-translational addition of sugar residues to the ECD and serves structural and functional roles in the CaR; it has been implicated in determining intracellular trafficking, cell-surface expression, facilitating protein folding and secretion (Fan et al., 1997). There are 11 predicted N-linked glycosylation sites on the ECD of human CaR. Nine of these are conserved between species; in rat, bovine, and chicken the amino acid sites asparagines (Asn-) 90, 130, 261, 287 446, 468, 488, 541 and 594 are all conserved (Ray et al., 1998). However Asn-386 is not present in bovine CaR and Asn-400 is not present in rat CaR. When human CaR underwent site-directed mutagenesis of Asn-594, -386 and -400 to glutamate, subsequent transient expression in HEK-293 cells did not affect the electrophoretic mobility of the transfected receptor in HEK-293 cells, inferring that these mutants are not efficiently glycosylated in this model. Site-directed mutation and transient expression in HEK-293 cells of Asn-468, Asn-488 and Asn-541 of the sites caused a decrease in receptor mass increasing electrophoretic mobility (Ray et al., 1998). Mutation of at least 5 sites, Asn-90, Asn-130, Asn-261, Asn-287 and Asn-446 caused severe loss of expression, most likely due to the endoplasmic reticulum retention of the proteins (Ray et al., 1998). There have been no clinical cases to date of mutations of N-linked glycosylation sites which may indicate that although such mutations may
reduce cell-surface CaR expression the effect in vivo may be less severe and thus sub-clinical.

Under reducing conditions in electrophoresis, the apparent molecular weights of CaR-immunoreactive bands are ~120, ~140 and ~160kDa which represent the non-glycosylated, high mannose and mature glycosylated forms of the receptor respectively (Bai et al, 1996). The 120kDa band corresponds to the predicted size of the CaR polypeptide and is rarely seen on western blots, except following glycosidase treatment (Bai et al, 1996), but was observed on immunoblots of renal tissue (Ward et al, 1998). The 140kDa high mannose protein represents the immature protein and is presumed not to reach the membrane (Bai et al, 1996), whereas the fully mature 160kDa appears to represent the functional CaR that does reach the cell membrane. Under non-reducing conditions, CaR immunoreactivity is also observed at ~280kDa and represents dimeric forms of the CaR (Bai et al, 1996; Bai et al, 1998a; Ward et al, 1998).

1.2.4 Dimerisation

It had previously been shown that mGluR5 exists on the cell membrane as a disulphide-linked homodimer (Romano et al, 1996). The dimerising region of mGluR5 was found to be in the first 17kDa region of the ECD, which contains four cysteine residues conserved between mGluR and CaR (Romano et al, 1996). As mentioned above, CaR also migrates electrophoretically as a dimer or higher order oligomer, i.e. ~240-310 kDa (Bai et al, 1998a; Ward et al, 1998). Although Cys-101 and Cys-236 were initially identified as essential for dimerisation, it was subsequently shown that Cys-129 and Cys-131 confer dimerisation (Pace et al, 1999; Ray et al, 1999). Studying both of these pairs of cysteines, Zhang et al found that mutating 129 and 131 abolished dimerisation whereas mutating cysteines 101 and 236 reduced expression but not dimerisation (Zhang et al, 2001).

Ray et al determined by site-directed mutagenesis that abolishing cysteines 129 and 131 resulted in a CaR that failed to dimerise but that was otherwise expressed normally and was still functional (Ray et al, 1999). Interestingly, mutating one or both cysteines rendered the receptor more sensitive to calcium and reduced its maximal response (Ray et al, 1999). Furthermore, human mutations adjacent to these cysteines exhibit gain-of-function appearing to confirm that homodimerisation actually lowers CaR sensitivity but to the physiologically-required degree that
provides for effective calcium homeostasis (Ray et al, 1999). There is some evidence that the CaR can form heterodimers with other family C GPCRs. CaR and mGluR1α were co-immunoprecipitated from bovine brain and can form disulphide linkages in HEK-293 cells but it remains to be confirmed whether this occurs in vivo or has any functional consequence (Chang et al, 2007; Gama et al, 2001).

1.2.5 Transmembrane domain

Hydropathy plot analysis of bovine CaR predicted seven membrane-spanning regions (Brown et al, 1993) and the human CaR TMD extends between amino acids 613 and 862 (Garrett et al, 1995). Interestingly, there are known mutations of the TMDs that cause both loss- and gain-of-function in CaR, indicating the functional importance of the TMDs (Hendy et al, 2003; Hu et al, 2005; Nagase et al, 2002; Nakajima et al, 2009; Shiohara et al, 2004; Watanabe et al, 1998). For example, the substitution mutation Ser-820-Phe (in the sixth transmembrane domain) elicited a gain-of-function, but did not alter cell surface expression in a HEK-293 cell model (Nagase et al, 2002). The transmembrane domains themselves can be divided into three; the extracellular loops, the intracellular loops and transmembrane-spanning regions.

Ray et al generated a series of truncated human CaRs in order to determine the importance of the TMDs and carboxyl terminus in determining expression (Ray et al, 1997b). CaR truncation at Thr-706 in the second intracellular loop and Thr-806 in the third intracellular loop resulted in a mutant protein that did not express in HEK-293 cells. A receptor truncated at Thr-865 which includes all seven transmembrane domains but not the carboxyl terminal had low level expression, but did not show any sensitivity to calcium (Ray et al, 1997b).

1.2.6 TMD extracellular loops

Studies using chimeras made from CaR and mGluR1 demonstrated that the transmembrane domains are critical for positive allosteric modulation by the calcimimetic R-568 (see Section 1.4.3) (Hauache et al, 2000a; Hauache et al, 2000b). Replacing only the cysteine-rich region with that from mGluR1 did not alter the ability of R-568 to increase receptor sensitivity; however, substituting the membrane-spanning domain abolished the effect of R-568 (Hauache et al, 2000b).
Hu et al then determined that mutating Glu-837 to alanine in the third extracellular loop caused a decrease in receptor sensitivity, and unlike other inactivating mutations, could not be augmented by R-568 (Hu et al, 2002). Mutating Glu-837 to the similar amino acid aspartate did not affect sensitivity of the receptor to either the negative allosteric modulator NPS-2143 (see 1.4.4) or the positive modulator R-568, whereas, its mutation to lysine abolished sensitivity to either NPS-2143 or R-568 (Hu et al, 2005).

Brown et al first demonstrated that bovine CaR contained an ELEDE motif of negative amino acids on the second extracellular loop (Brown et al, 1993). The motif is also found in human CaR-spanning amino acids 755-759 (Garrett et al, 1995). Studying the effect of mutating the ELEDE motif in the second extracellular loop Hu et al discovered that mutating Glu-767, Asp-758 or Glu-759 increased the sensitivity of the CaR (Hu et al, 2002). There are a number of possibilities for this observation; it is possible that the acidic residues maintain the receptor in an inactive confirmation and mutation facilitates activation, or, that they are directly involved in calcium binding. Ray et al also found that mutating Glu-767 to alanine caused a gain-of-function mutation and had a significantly lowered CaR EC$_{50}$ (for PI hydrolysis) from 4.2 mM to 2.1 mM (Ray et al, 2004). However, mutating Asp-758 or Glu-759 did not affect EC$_{50}$ (Ray et al, 2004). However, there are no known novel mutations in this region causing either FHH or ADH; therefore the mutation of residues in this region may not be significant in vivo.

1.2.7 TMD Intracellular loops

To determine if amino acids in the TMD intracellular loops are vital for signalling, Chang et al sequentially mutated individual amino acids within the second and third intracellular loops (ICLs) (Chang et al, 2000). Mutating Phe-707 to alanine significantly reduced the ability of the receptor to activate PLC in HEK-293 cells, whereas mutating neighbouring amino acids did not (Chang et al, 2000). Interestingly, mutating Phe-707 to the similar amino acid tryptophan restored some function to approximately 50% that of wild-type (WT) (Chang et al, 2000). Human CaR shares 67-85% homology with the mGluRs, in the third intracellular loop (Chang et al, 2000). Mutation of Leu-798, Phe-802 and Glu-804 to alanine, in the third ICL, caused significantly reduced receptor activity. Interestingly mutation of Leu-798 to the closely related amino acid isoleucine restored receptor activity comparable to that of WT, whereas unrelated amino acid substitution did not restore
activity (Chang et al., 2000); inferring that the hydrophobic properties of this residue are critical for activity. Substitution of Phe-802 to the related tyrosine again restored activity and substitution of Glu-804 to aspartate or glycine produced results comparable to WT (Chang et al., 2000). Expression was unchanged for Leu-798-Ala and Phe-802-Ala but there was no detectable band at 160 kDa for Glu-804-Arg (Chang et al., 2000).

1.2.8 Intracellular domain

The ICDs represent the most diverse regions of the class C GPCRs, which allows for greater receptor-specific control of the various downstream signalling pathways. There are also relatively few clinical cases of ICD mutations resulting in either gain- or loss-of-function. Indeed, there are three known polymorphisms in the ICD which are not associated with any significant pathology, which supports the idea that great amino acid variability is possible in the ICD than elsewhere without a resultant loss of receptor function (Eren et al., 2009; Perez-Castrillon et al., 2006; Rubin et al., 1997; Toke et al., 2007; Vezzoli et al., 2007). Truncations of the Mozambique tilapia CaR show that approximately 100 amino acids in the ICD can be removed and the receptor still retains PLC activity and ERK activation (Loretz et al., 2004). Ray et al. showed that truncation of human CaR at 865, or 874, caused a decrease in cell surface expression and loss of PLC activation (Ray et al., 1997b). One group did find that truncation at 866 was expressed, but it was necessary to transfect 10 times the amount of DNA for comparable expression levels, of bovine CaR (Chang et al., 2001). However, truncation at Thr-888 had expression and PI hydrolysis comparable to WT (Ray et al., 1997b) this is compounded by Gama and Breitwieser who found that truncation at 886 did not affect Gαq/11-mediated signalling (Gama & Breitwieser, 1998). Therefore, there must be function-critical residues between 874 and 888, indeed further site-directed mutagenesis showed that substitution of 881-883 with 3 alanine residues reduced CaR expression and decreased its maximal response by 60-70% compared to WT. Mutation of human CaR residues 875-879 to alanine reduced expression and abolished sensitivity to Ca2+ up to 20 mM, indicating an amino acid in this region is critical for PLC activation (Ray et al., 1997b).

Chang et al. used tandem-alanine mutations in bovine CaR to determine the residues responsible for expression and PLC activation respectively (Chang et al., 2001). Mutating 879-883 to alanine abolished PLC activation in full length bovine CaR but did not affect expression. Single site-directed mutation of His-880 and Phe-882
reduced activation of PLC to 17% and 35% of WT response (Chang et al, 2001).
Immunohistochemistry showed that although these mutants were translated they
were not expressed at the membrane.

There are five putative PKC phosphorylation sites in human CaR (Garrett et al,
1995), including Thr-646 on the first intracellular TMD loop and Ser-794 on the
third intracellular loop. The remaining three, Thr-888, Ser-895 and Ser-915, are
located on the ICD close to the juxta-membrane region. Mutation of Thr-646 and
Ser-794 to valine and alanine respectively, did not affect the EC$_{50}$ of the receptor
(Bai et al, 1996; Bai et al, 1998b). Mutation of either Ser-895 or Ser-915 to alanine
did elicit marginal increases in Ca$^{2+}$ sensitivity compared to WT however, the
mutation with the greatest effect by far, was the substitution of Thr-888 to valine
which resulted in a significant increase in CaR Ca$^{2+}$ sensitivity an effect that was not
significantly additive when co-mutated with either S895A or S915A (Bai et al,
1998b). Expression was not compromised for any of the mutations. Furthermore,
acute treatment of WT CaR with the PKC activator PMA caused a right-ward shift in
receptor sensitivity. However, in the mutation Thr888Val Ca$^{2+}$ sensitivity was partly
inhibited and EC$_{50}$ was increased from 2.9 mM to 3.3 mM, for Ca$^{2+}$ (Bai et al,
1998b). Phosphorylation of Thr-888 will be discussed in detail later. Furthermore,
there are also 2 putative PKA sites in the ICD at Ser-899 and Ser-900 and these will
also be discussed fully later.

1.3 Calcium-sensing receptor tissue expression

The major physiological function of the CaR is the central coordination of
extracellular free ionised calcium homeostasis, primarily via a suppression of PTH
secretion. However, its expression in other tissues has been widely described. Initial
Northern blot analysis showed that CaR was expressed not only in the parathyroid
but also in a wide variety of tissues including the brain, thyroid and kidney (Aida et
al, 1995; Brown et al, 1993; Riccardi et al, 1995). The CaR is functionally a calcium
sensor however, it is reported that CaR can ‘sense’ other cations and L-amino acids
(Conigrave et al, 2000). The receptor has since been identified in calcitropic and
non-calcitropic tissues such as the gastrointestinal tract, blood vessels, bone and
cartilage, breast tissue, spermatozoa and epidermis (Chang et al, 1999; Cheng et al,
1998; Dell’Aquila et al, 2006; House et al, 1997; Kameda et al, 1998; Mendoza et al;
Ray et al, 1997a; Weston et al, 2008). A great number of functional effects have been
ascribed to the CaR; some of these will be detailed below.
1.3.1 Parathyroid gland

First postulated to be expressed in the parathyroid gland (López-Barneo & Armstrong, 1983), CaR expression is fundamental to ensure calcium homeostasis. CaR is expressed on the chief cells and localised to caveolae (Chow et al; Kifor et al, 1998). The CaR inhibits PTH secretion, through a currently unknown mechanism, thereby mediating calcium excreted by the kidneys, stored in bone and absorption from the gut (indirectly). CaR expressed in the gut, kidney and bone also contributes to calcium homeostasis (Figure 1.1). The CaR also contributes to the suppression of parathyroid chief cell hyperplasia; homozygous loss-of-function in the CaR is associated with parathyroid gland hyperplasia [reviewed in (Bai, 2004)]. Furthermore, culturing bovine parathyroid cells cultured ex vivo, is associated with a loss of CaR expression; which was synonymous with a decrease in CaR mRNA (Brown et al, 1995). Interestingly, it is an increase in intracellular cAMP which drives PTH secretion (Shoback et al, 1984); here the relationship between cAMP and the CaR is studied in detail (see Chapter 4).

![Figure 1.1. Schematic overview of extracellular free ionised calcium homeostasis.](image)

Parathyroid hormone (PTH) released by the parathyroid glands acts on bone and the kidney to raise plasma free ionised calcium levels. Furthermore, the kidney catalyses the formation of the active form of 1,25 dihydroxyvitamin D₃, which acts on the gut to raise plasma free-calcium levels.
The CaR is found throughout the kidney nephron, in both the cortical and medullary tubules. The CaR is expressed on the proximal convoluted tubule (PCT) apical membrane, ascending loop of Henle basolateral membrane, apical and basolateral membranes of the distal convoluted tubule (DCT) and cortical collecting duct (CCD) and the apical membrane of the medullary collecting duct (MCD) (Riccardi et al, 1998; Riccardi et al, 1996). The widespread expression is indicative of the importance of CaR expression in the kidney. Indeed, the CaR contributes to the maintenance of phosphate homeostasis, urinary acidification and concentration and mineral ion transport [reviewed by (Riccardi & Brown, 2010)].

In the PCT, calcium feedback controls CaR and vitamin D₃ activation. High luminal Ca²⁺ concentration activates the CaR and has been reported to suppress 1α-hydroxylase, which is the enzyme responsible for 1,25 dihydroxyvitaminD₃ generation (Bland et al, 1999; Maiti et al, 2008). These interactions show that calcium homeostasis is capable of fine-tuning and local control of calcium concentration.

The CaR also regulates Ca²⁺ reabsorption in the cortical thick ascending loop of Henle (cTAL) (Figure 1.2). There, sodium is reabsorbed with potassium and two chloride ions via the NKCC2 transporter, across the apical membrane (Hebert et al, 1997). Chloride leaves the cTAL cells across the basolateral membrane through chloride channels, while the potassium is returned to the lumen through K⁺ channels (ROMK) (Gamba & Friedman, 2009; Ward & Riccardi, 2002). Na⁺ is then pumped out of the cell, against the gradient, across the basolateral membrane via the Na⁺-K⁺ ATPase (Wang et al, 1997). Thus, reabsorption of Na⁺ is the driving force for Ca²⁺ paracellular reabsorption by generating a lumen-positive transepithelial gradient, down which Ca²⁺ and Mg²⁺ can move back into to the interstitium via a paracellular route (Hebert et al, 1997; Ward & Riccardi, 2002). High plasma Ca²⁺ concentration will then activate the CaR on cTAL causing it to inhibit ROMK on the apical membrane slowing the recycling of K⁺ therefore providing less luminal K⁺ for NKCC2 and thus limiting the availability of Na⁺ to generate the lumen-positive gradient (Wang et al, 1997; Wang et al, 1996; Ward & Riccardi, 2002). When luminal Ca²⁺ concentration is high, inhibiting reabsorption further would minimise the risk of kidney stone formation (Brown & Hebert, 1995; Hebert et al, 1997).

CaR may also act by limiting cAMP and therefore AVP-stimulated divalent ion uptake (Desfleurs et al, 1998). Paracellular Mg²⁺ transport requires claudin-16, an
integral gap-junction protein (Ikari et al, 2008). In Madin-Darby canine kidney (MDCK) cells activation of CaR induces claudin-16 degradation therefore also regulating Ca$^{2+}$ and Mg$^{2+}$ transport (Günzel et al, 2009; Ikari et al, 2008). NKCC2 is also able to transport NH$_4^+$ in substitution for K$^+$ across the apical membrane (Amlal et al, 1994; Farajov et al, 2008). Protons then leave the cell across the apical membrane via NHE or anion exchange (Capasso et al, 2002; Good et al, 2004).

In the DCT, CaR is present on both the apical and basolateral membranes (Riccardi et al, 1998). High urinary Ca$^{2+}$ concentrations in the DCT activates the CaR on the apical membrane opening epithelial Ca$^{2+}$ channel transient receptor potential vanilloid member 5 (TRPV5) channels allowing calcium to be reabsorbed across the apical membrane (Topala et al, 2009). Calcium is chaperoned across the cell via calbindin D28K and is extruded across the basolateral membrane via the plasma membrane Ca$^{2+}$-ATPase (PMCA) and Na$^+$/Ca$^{2+}$ exchanger (NCX) (Hoenderop et al, 2002). Activation of CaR on the basolateral membrane inhibits PMCA, inhibiting Ca$^{2+}$ extrusion (Hoenderop et al, 2002).
**Figure 1.2. Schematic of mineral reabsorption in the cortical thick ascending limb.** The water impermeable thick ascending limb (cTAL) allows calcium and magnesium reabsorption via the generation of a lumen-positive transepithelial gradient (Hebert et al, 1997). The electroneutral bumetanide-sensitive NKCC2 reabsorbs Na⁺, K⁺ and 2Cl⁻, the Na⁺ and Cl⁻ exit via the Na⁺/K⁺ ATPase and Cl⁻ channels respectively (Hebert et al, 1997). The K⁺ ions are recycled across the apical membrane via the ROMK, generating the lumen-positive gradient. Calcium and magnesium and water are reabsorbed paracellularly. Activation of the CaR causes cPLA2 mediated AA production, which inhibits the ROMK, inhibiting further calcium reabsorption (Wang et al, 1997; Wang et al, 1996).

The CaR is expressed on both the apical and basolateral membranes in the cortical collecting duct (CCD) where it again colocalises with TRPV5 channels (Renkema et al, 2009). TRPV5 knockout mice exhibit polyuria and urinary acidification when exposed to CaR agonists (Renkema et al, 2009). This suggests that CaR protects against nephrolithiasis. In the inner medullary collecting duct (IMCD), CaR colocalises with aquaporin2 (AQP2), and its activation inhibits insertion of AQP2, decreasing water reabsorption (Procino et al, 2004; Ward et al, 1999).
1.3.3 Bone

Bone acts as a reservoir for calcium and is able to respond to fluxes in plasma calcium concentration rapidly and independently of PTH secretion (Dvorak & Riccardi, 2004). Chronic calcium imbalance, such as hypocalcaemia, can lead to bone remodelling and demineralisation. Expression of CaR has been widely reported in a number of osteoblast cell lines and human bone [reviewed in (Brown et al, 2004; Yamaguchi, 2008)]. Chang et al have shown that in an osteoblast cell line, increasing in $\text{Ca}^{2+}_o$ cause IP$_3$ and $\text{Ca}^{2+}_i$ increase (Chang et al, 1999). The possible functional role of CaR in osteoblasts was previously challenged by data showing that osteoblasts from CaR knockout mice retain their $\text{Ca}^{2+}_o$-sensitivity (Pi et al, 1999; Quarles et al, 1997). However, Chang and co-workers generated an osteoblast-specific CaR null mouse that exhibits a severe bone phenotype (Chang et al, 2008). This group further showed that the original CaR knockout is a hypomorph and may retain some function which could explain the observations of Quarles and co-workers (Brown & Lian, 2008).

1.3.4 Gastrointestinal system

CaR expression has been reported throughout the GI tract from the oesophagus to the colon (Bruce et al, 1999; Chattopadhyay et al, 1998; Justinich et al, 2008; Rutten et al, 1999) where it is a vital component in nutrient sensing and the secretory mechanism. As well as divalent cations, the CaR is also able to sense L-amino acids (see Section 1.4.2) (Conigrave et al, 2000), suggesting that CaR may have a broader role in nutrient sensing. CaR expressed on the basolateral membrane of parietal cells causes proton extrusion from the cells by activation of $\text{H}^+/$K$^+$ ATPase (Busque et al, 2005; Remy et al, 2007). Therefore, L-amino acid and cation concentrations in the plasma may jointly contribute to acid secretion in the GI tract (Conigrave & Brown, 2006). CaR may also modulate secretion by gastrin-secreting cells, where it is expressed on both the apical and basolateral membranes (Buchan et al, 2001; Dufner et al, 2005; Ray et al, 1997a). In the stomach, CaR has been found on gastrin-secreting cells, but not somatostatin- or mucin-secreting cells (Cheng et al, 1999). Indeed, high $\text{Ca}^{2+}_o$ concentration, or spermine exposure, causes a dose-dependent increase in gastric acid secretion (Ray et al, 1997a).

Interestingly, the CaR is also expressed on the tongue where it has been suggested that the receptor forms heterodimers with the taste receptors (Conigrave &
Hampson, 2006; San Gabriel et al, 2009). Indeed, there is evidence that the CaR may contribute functionally to the enhancement of certain tastes (Maruyama et al, 2012; Ohsu et al). In the pancreas, CaR is expressed in rat both in the acinar and exocrine ductal cells where it has been suggested that CaR functions to reduce lithogenesis, a major cause of pancreatitis (Bruce et al, 1999), possibly by altering bicarbonate fluid secretion. This relates to the fact that high Ca$^{2+}$ concentrations in the ductal regions along with high bicarbonate concentrations can lead to the formation of calcium stones and blockage of the pancreatic ducts. CaR is also expressed on the apical membrane of the ductal cells where it may sense increasing Ca$^{2+}$ concentrations and stimulate fluid secretion leading to the dilution of ductal fluid reducing the likelihood of stones formation (Bruce et al, 1999). The fact that the ductal region is also alkali, due to the high bicarbonate concentrations, might be favourable as Quinn et al found that CaR is more sensitive alkali fluid (see Section 1.4.5) (Quinn et al, 2004).

1.3.5 Central and peripheral nervous system

Northern blot analysis of bovine samples revealed CaR expression in the cerebral cortex and cerebellum (Brown et al, 1993) and in situ hybridisation showed that the CaR is widely expressed throughout the rat brain (Rogers et al, 1997). Indeed, it is now well established that the CaR is expressed throughout the brain and central nervous system (Yano et al, 2004). Interestingly, the highest density of CaR expression was found in the subfornical organ (SFO), an area critical for electrolyte balance and modulation of drinking behaviour (Rogers et al, 1997). Washburn et al showed that spermine and R-467, but not S-467, caused neuronal depolarisation in rat subfornical organ (Washburn et al, 2000; Washburn et al, 1999). The SFO is circumventricular, meaning it is outside the blood-brain barrier and therefore exposed to circulating molecules (Washburn et al, 1999). Thus, hypercalcaemia could activate the CaR inducing thirst and therefore prevent dehydration (Yano et al, 2004).

Chattopadhyay et al showed that CaR expressed in rat hippocampus was up-regulated 10-30 days postnatally and then its expression declined to the levels found in adult rat (Chattopadhyay et al, 1997). This postnatal increase suggests a role for CaR in development and it was hypothesised that CaR could mediate long-term potentiation (LTP) (Chattopadhyay et al, 1997). One possible mode of action could be via K$^+$ channels; Vassilev et al found that CaR agonists increase the open
probability of K⁺ channels in hippocampal neurons in WT mice but not homozygous CaR knockout mice (Vassilev et al., 1997). Interestingly, amyloid-β peptides (Aβ) protein can increase CaR activity in CaR-HEK cells and mice hippocampal neurons (Ye et al., 1997). This indicates that CaR could be involved in Alzheimer's disease (AD) progression and degenerative loss of memory and cognitive function. Furthermore, CaR has been associated with an increase in AD susceptibility, particularly in patients without an APOE4 allele (Conley et al., 2009).

1.3.6 Blood vessels

A role for CaR in blood vessel contraction was first proposed by Bukoski and co-workers and supported by data from Ohanian et al (Bukoski et al., 1995; Ohanian et al., 2005). CaR was shown to be expressed on the vascular endothelial cells of rat mesenteric arteries where activation by the calcimimetic, Calindol, causes hyperpolarisation of the cells by opening Ca²⁺-sensitive small and intermediate conductance potassium channels (SKCa and IKCa) (Weston et al., 2005). These findings have been corroborated by immunoblot, immunoprecipitation of CaR and IKCa and knocking down the expression of CaR with siRNA (Weston et al., 2005). There is also evidence of CaR expression in the smooth muscle cells underlying the endothelium, and it has been hypothesised that such expression helps preserve the SMC phenotype (Alam et al., 2009). Indeed, vascular calcification is associated with decreased CaR expression in the vessels (Alam et al., 2009). Furthermore, in cultured VSMCs, over-expression of a dominant negative CaR increases mineralisation whereas calcimimetics ameliorate it (Alam et al., 2009). This could be of potential clinical importance in light of the possible benefit of calcimimetics against vascular calcification in rat models of chronic kidney disease (Ivanovski et al., 2009; Kawata et al., 2005). Disappointingly, the recent EVOLVE study only detected a non-significant 7% relative reduction in the incidence of major cardiovascular events following calcimimetic therapy in renal dialysis patients enlisted on an intention-to-treat trial. However, 23% of the placebo group were also given commercial Cinacalcet and the trial did report a significant decrease in the risk of calciphylaxis which represents one of the most life-threatening complication of kidney disease (Perkovic & Neal, 2012).
1.3.7 Developing lung

Studies have shown that high extracellular calcium inhibits lung development in mouse embryos (Finney et al, 2008). Explanted lung tissue from E12.5 mice, were grown in culture in low (1.05 mM) or high (1.7 mM) Ca\textsuperscript{2+}; exposing the tissue to low calcium increased branching whereas high calcium or calcimimetic R-568 had significantly less branching (Finney et al, 2008). Activation of CaR significantly decreases proliferation, and therefore branching, in mouse embryonic lung tissue (Finney et al, 2008). There was no significant difference in body or lung weight between WT and (-/-) mice embryos (Finney et al). Interestingly, although full length CaR was not expressed in the homozygous knockout mouse, an exon 5 splice variant mRNA was detected by RT-PCR (Finney et al, 2011). This may compensate for the lack of full length CaR and prevent a more severe phenotype. The exon 5 splice variant is able to sense agonists such as Ca\textsuperscript{2+} and neomycin similarly to the WT CaR, but is only expressed when in the absence of full-length receptor (Finney et al, 2011; Riccardi et al, 2009).

1.3.8 Other tissues

In addition, CaR expression has also been described in a variety of other tissues including mammary gland, keratinocytes, haematopoietic cells, spermatozoa and oocytes (Cheng et al, 1998; Dell’Aquila et al, 2006; House et al, 1997; Mendoza et al; Oda et al, 1998; VanHouten et al, 2004). During pregnancy and lactation mRNA levels of CaR increase in the mammary gland and decrease following weaning (VanHouten et al, 2004). Heterozygous knockout mice (+/-) also had decreased CaR mRNA and decreased calcium content in their milk (Ardeshirpour et al, 2006). This evidence suggests that during pregnancy and lactation the mammary gland becomes capable of sensing extracellular calcium.

1.4 Calcium-sensing receptor pharmacology

1.4.1 Orthosteric ligands

Although termed the calcium-sensing receptor, CaR is also capable of sensing a broad range of ligands including di- and trivalent cations, L-amino acids and polyamines (Brauner-Osborne et al, 2007). The CaR has a relatively modest Ca\textsuperscript{2+}o sensitivity in the low millimolar range, with an EC\textsubscript{50} of approximately 1.2 mM in vivo.
(Brown & MacLeod, 2001). This represents one of the highest EC$_{50}$ values for any GPCR responding to its primary agonist, but appears ideal physiologically given the need for maintenance of free-ionised calcium concentrations $\sim$1.2 mM (Brown & MacLeod, 2001). The CaR tends to be maximally activated at Ca$^{2+}$o concentrations $\approx$5 mM and the steepness of the relationship is indicated by the Hill coefficient for Ca$^{2+}$o concentration of 3-5, which permits the Ca$^{2+}$o-mediated control of PTH secretion over a very narrow range (Brown & MacLeod, 2001).

Interestingly, the order of agonist potency for inositol metabolism in bovine parathyroid cells, reveals that calcium is not the most potent cation, the order of potency is as follows; Gd$^{3+}$ $>$ La$^{3+}$ $>$ Ca$^{2+}$ $=$ Ba$^{2+}$ $>$ Sr$^{2+}$ $>$ Mg$^{2+}$ (Handlogten et al, 2000). The potency of the cationic ligands for the CaR depends on two factors; the charge of the ion and the ionic radii (Quinn et al, 1997). For ions with the same charge those with a greater radius have a greater potency and for ions of a similar size, those with a greater charge have a greater potency (Chang & Shoback, 2004; Quinn et al, 1997).

Produced in the gut and synaptic cleft in vivo, polyamines are potent orthosteric agonists of the CaR. The most potent is spermine which has an EC$_{50}$ for Ca$^{2+}$o-mediated CaR activation of 300 µM (Quinn et al, 1997). Spermidine exhibits an EC$_{50}$ of 4 mM, whereas putrescine did not activate CaR at concentrations less than 10 mM (Quinn et al, 1997). Potency is linked to the number of amine groups in the ligand; spermine has the most at 4, with spermidine having 3 and putrescine only 2 (Chang & Shoback, 2004; Quinn et al, 1997). Therefore, like cations where the charge ratio determines potency, the number of amine groups can also determine potency.

### 1.4.2 CaR allosteric modulators – aromatic amino acids

The calcimimetic agent NPS-R568 (see 1.4.3) is structurally similar to tyrosine and for this reason Conigrave et al investigated the effect of amino acids on CaR activation (Conigrave et al, 2000). Conigrave and co-workers found in stably transfected CaR-HEK cells, that whereas moderately low Ca$^{2+}$o concentrations (~1.5 mM) did elicit some Ca$^{2+}$i mobilisation, addition of some L-amino acids potentiated these responses (Conigrave et al, 2000). It has also been shown that the L-amino acids modestly potentiate ERK phosphorylation as well (Conigrave et al, 2007; Lee et al, 2007).

This effect of amino acids was stereoselective; L-amino acids were more potent than D-amino acids (Conigrave et al, 2004). In 2.5 mM Ca$^{2+}$o the potency of amino acids
was $L$-Phe $= L$-Trp $= L$-His $\geq L$-Ala $\geq L$-Ser $= L$-Pro $= L$-Glu $\geq L$-Asp $L$-Phe (Conigrave et al., 2000). The L-amino acids could also shift the concentration effect curve for calcium; for example 10 mM L-Phe reduced the $EC_{50}$ of $Ca^{2+}$ from 4.2 mM to 2.2 mM (Conigrave et al., 2000). Aromatic and aliphatic amino acids were more effective than positive branched amino acids (Conigrave et al., 2002). L-amino acids require a threshold $Ca^{2+}$ concentration to activate the receptor however, this is not uniform; L-Ala is able to elicit responses in 0.5 mM whereas L-Phe requires 0.75 mM (Conigrave et al., 2007).

The same group identified that L-amino acids bind to the VFD domain or exert their influence through the VFD and do not require the cysteine-rich domain, transmembrane domain or C-terminus (Mun et al., 2004). Sequence analysis between the CaR and mGluR1 and analysis of the crystalline structure of mGluR1 showed that there are a number of conserved amino acids (Mun et al., 2005). Ser-169, 170 and 171 had previously been identified as critical for L-amino acid sensing (Zhang et al., 2002b), in addition to this residue the group identified Thr-145 as being vital for L-amino acid-sensing (Mun et al., 2005). Mutation of either Ser-170 or Thr-145 residue abolished or reduced CaR sensitivity to changes in L-Phe and interestingly Thr-145-Ala abolished the receptor’s stereoselectivity (Mun et al., 2005).

1.4.3 Allosteric CaR activators - Calcimimetics

Before the identity of the CaR was identified, the search for an agonist had already begun and only two years after the receptor was cloned, the discovery of the calcimimetics R-568 and R-467 were published (Kronenberg & Fischer, 1995; Nemeth, 2006; Nemeth et al., 1998). Calcimimetics are phenylalkylamines and are only effective in the presence of $Ca^{2+}$. They reduce the calcium concentration necessary to elicit a response, causing a leftward shift for the concentration-effect curve. The calcimimetics, NPS-467 and NPS-568 are stereoselective; the S-enantiomer is ten to a hundred times less potent than the R-enantiomer (Hammerland et al., 1998; Nemeth et al., 1998). Furthermore, R-568 inhibits PTH release from bovine parathyroid cells dose-dependently (Nemeth et al., 1998). The phenylalkylamines bind to a distinct site on the CaR to that of $Ca^{2+}$; Hu et al identified Glu-837, at the top of the seventh TMD and third extracellular loop, as critical for R-568 sensitivity (Hu et al., 2002; Zhang et al., 2002a). Calcimimetics have also been found to be able to rescue calcium responsiveness in previously
unresponsive CaR mutants; Zhang et al hypothesised that these compounds increase receptor affinity for G proteins or increase downstream signalling (Zhang et al, 2002a). Due to its ability to limit PTH secretion, NPS-1493, or Cinacalcet (US), soon became an attractive prospect as a drug for hyperparathyroid conditions. It is now licensed as a treatment for secondary hyperparathyroidism and parathyroid cancer (Amgen, 2003; Nemeth et al, 2004).

1.4.4 Allosteric CaR inhibitors - Calcilytics

Sustained serum PTH levels are catabolic for bone formation, causing gradual but potentially pronounced demineralisation such as in primary hyperparathyroidism (Nemeth, 2002). In contrast, periodic or pulsatile PTH secretion is more physiological and causes anabolic bone turnover. The only way to harness this dual effect of PTH therapeutically at present is via daily PTH injections which is expensive and has compliance issues. Therefore, the need to develop a compound which could enhance physiological PTH release is significant. In contrast to the calcimimetic compounds which cause the receptor to become more sensitive and shift the concentration-effect curve leftward, the calcilytics inhibit the CaR’s sensitivity to Ca\(^{2+}\) i.e. shifting the concentration-effect curve to the right (Nemeth, 2002). High throughput screening yielded compounds which are structurally similar to the calcimimetics. One of the first calcilytics discovered was NPS-2143. It was one of three such compounds discovered by high-throughput screening and chosen for its activity and selectivity; indeed it failed to inhibit other class C GPCRs. In stably transfected CaR-HEK cells, NPS-2143 causes a dose-dependent rightward shift in the Ca\(^{2+}\) concentration-effect curve, without affecting maximal response (Nemeth et al, 2001). Consistent with this, NPS-2143 also dose-dependently increased PTH secretion from bovine parathyroid cells and infused rats (Nemeth et al, 2001).

Unfortunately, NPS-2143 and its analogue Ronacaleret have not passed clinical trials for use in osteoporosis and bone repair due to other interactions and a lack of efficacy respectively. At the present time GlaxoSmithKline are beginning clinical trials using Ronacaleret to determine its affect at mobilising haematopoietic (CDC34\(^+\)) stem cells (GlaxoSmithKline, 2013).
1.4.5 Effect of extracellular pH on CaR

Modulation of receptors, channels and bacteria by protons are well documented (Bechinger, 1996; Chokshi et al, 2012; Clarke et al, 2000; Geierstanger et al, 1998; Horng et al, 2005; Muller et al, 2009; Zong et al, 2001). The interaction between pH and the parathyroid has been documented and speculated about for some time (Batlle et al, 1980; Chan & Bartter, 1980; Coe et al, 1975; Massry et al, 1974). Experiments in dogs show that metabolic acidosis significantly increases PTH release (Lopez et al, 2002). Interestingly, blood pH decreased by only 0.1 pH units significantly increase PTH secretion (Lopez et al, 2004). The reverse is also true with induced metabolic alkalosis in dogs significantly inhibiting PTH secretion independent from ionised calcium (Lopez et al, 2003).

Quinn et al showed, using a heterologous expression system, that non-physiological changes in pH$_o$ significantly alter the potency of the CaR receptor for Ca$^{2+}$o and Mg$^{2+}$o (Quinn et al, 2004). In acidic conditions (pH 6.5), CaR sensitivity was reduced, as measured by Ca$^{2+}$i mobilisation. The reverse was true in alkaline conditions (pH 8.5) where the sensitivity of the receptor was increased (Quinn et al, 2004). Comparing concentration-effect curves showed that increasing pH$_o$ correlated with a left-ward shift of the Ca$^{2+}$o curves. These results are corroborated by Doroszewicz et al who used Xenopus laevis oocytes, in a dual expression of the CaR and the calcium-activated potassium channel (SK4) to measure activation (Doroszewicz et al, 2005). As the activation of SK4 is not directly proportional to the activation of CaR, the threshold Ca$^{2+}$o concentration necessary to elicit K$^+$ currents was analysed. In 1.8 mM Ca$^{2+}$o significantly higher currents were observed in basic conditions (pH 8) than in acidic conditions (pH 6.5) (Doroszewicz et al, 2005). Comparing normalised currents, the K$_m$ value, for half-maximal activation, was significantly reduced in alkali conditions.

Previous experiments performed in this laboratory have shown that smaller, pathophysiological pH$_o$ changes (± 0.2 units) significantly altered CaR-induced Ca$^{2+}$i mobilisation in CaR-HEK cells and bovine parathyroid cells (Figures 1.3 and 1.4 respectively). Sensitivity of the CaR to pH$_o$ was also observed for other readouts of receptor activity. Thus, for example alkalosis potentiated ERK phosphorylation at a single Ca$^{2+}$o concentration with acidosis inhibiting ERK phosphorylation. CaR activation also causes the activation of Rho leading to actin polymerisation in CaR-HEK cells (Davies et al, 2006). Thus, elevating pH$_o$ by 0.2 units potentiated the increase in actin polymerisation, with lowered pH$_o$ (7.2) inhibiting it. To further
demonstrate that this effect is due to the changes in CaR agonist sensitivity, as opposed to a change in intracellular pH (pH$_i$), cells were loaded with the intracellular dye 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein (BCECF). Subsequent challenge of the CaR-HEK cells with either +0.4 or -0.4 pH$_o$ changes did not affect pH$_i$ over the acute timescales tested (McCormick, 2008).

Figure 1.3. Effect of pH$_o$ increase on CaR-induced Ca$^{2+}$ mobilisation in CaR-HEK cells. Ai and ii) Representative trace showing Ca$^{2+}$ changes (Fura2 ratio) in 2 single cells (orange, blue) or “global” cluster of cells (black) in response to elevated [Ca$^{2+}$]$_o$ (2.5 vs 0.5 mM control) at pH$_o$ 7.2 (ii) or 7.6 (i). Bi and ii) Quantification of these changes presented as area under the curve / min. **P<0.01 *P<0.05 vs. first pH 7.4 treatment by paired t-test, N=4. Presented with the permission of Dr W. McCormick (McCormick, 2008).
Figure 1.4. Effect of pH\textsubscript{o} increase on CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation in bovine Parathyroid chief cells. Ai and ii) Trace showing Ca\textsuperscript{2+}\textsubscript{i} changes (Fura2 ratio) in 2 single cells (orange, blue) or “global” cluster of cells (black) in response to elevated [Ca\textsuperscript{2+}]\textsubscript{o} (2.5 vs 0.8 mM control) at pH\textsubscript{o} 7.2 (ii) or 7.6 (i). Bi and ii) Quantification of these changes shown as area under the curve / min. N=3. * = P<0.05 vs. initial pH 7.4 response by paired t-test. Presented with the permission of Dr W. McCormick (McCormick, 2008).

Histidine has been implicated as the residue responsible for pH sensitivity in a number of receptors, channels and bacteria, including the mGluRs (Levinthal et al, 2009). Although normally associated with disulphide bond formation and posttranslational modification, cysteine also has a pKa within the physiological
range and therefore a candidate for determining pH sensitivity (Quinn et al, 2004). The human CaR contains 16 extracellular histidines; 15 in the ECD and one in the second extracellular loop of the transmembrane domain (Table 1).

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Table 1. CaR extracellular histidine conservation. Of the sixteen extracellular histidines all are conserved between species shown with the exception of His-254 which is found only in human CaR and His-595 which not present in mouse.

The histidines in the ECD were modelled (Dr Jim Warwicker, University of Manchester) using PyMOL software (Figure 1.5). The mutated histidine and cysteine CaRs were generated by site-directed mutagenesis (see chapter 2 Materials and Methods). Having validated the mutation by sequencing the plasmid they were transiently transfected into wild-type HEK293 cells first validated by ascertaining their EC$_{50}$ and secondly to determine if they retained pH sensitivity (see Chapter 3).
**1.5 Diseases of the calcium-sensing receptor and parathyroid gland**

1.5.1 Loss-of-function CaR mutations

Familial hypercalcaemia hypocalciuria (FHH) is caused by a loss-of-function mutation to the CaR gene. There are over 100 documented accounts of mutations causing FHH found along the whole length of the protein (Hendy, 2003; Hendy et al, 2009) and many of these have been catalogued on-line (Hendy, 2003).

The condition is either hereditary autosomal dominant, or it arises from a dominant *de novo* mutation (Alvarez-Hernandez et al, 2003). Patients usually present with moderate hypercalcaemia and one or both of inappropriately low calciuria and...
moderately high / inappropriately normal PTH levels. Subsequent genotyping is usually necessary to confirm the diagnosis (Heath, 1998). FHH can go unrecognised and the carrier may be asymptomatic, the severity of the disease depends upon the location of the mutation in the CaR gene (Hendy et al, 2009). Most mutations occur on the CaR locus on chromosome 3 however, some cases of FHH in families have been found on chromosome 19, the so-called Oklahoma variant (Lloyd et al, 1999). Also known as FHH type-3, the genetic cause of this condition has recently been ascribed to mutations in adaptor protein-2 (AP2) which is a key component of clathrin-coated vesicles (CCVs) as required for clathrin-mediated endocytosis (Nesbit et al, 2013).

Loss-of-function CaR mutations can manifest functionally in a number of ways; decreased expression of the CaR, decreased sensitivity of the receptor, disrupted intracellular signalling pathways or decreased transcription (Hendy et al, 2000). However, they all have the same outcome, reduced inhibition of PTH secretion. In support of this, heterozygous CaR knockout (-/+ ) mice exhibit a similar phenotype to FHH. That is, they exhibit inappropriately high PTH levels and relative hypocaliuria resulting in elevated blood ionised calcium levels (Ho et al, 1995).

Homozygous inheritance of loss-of-function CaRs can result in neonatal severe hyperparathyroidism (NSHPT) and can be fatal at birth without surgical intervention [reviewed (Hendy et al, 2009)]. Normally due to consanguineous relations between two carriers of FHH, NSHPT can also be caused by two unrelated FHH genes, or a de novo mutation in an individual with FHH (Hendy et al, 2009). Infants may suffer from weakened bones prone to fracture and neurodevelopment problems (Hendy et al, 2000; Hendy et al, 2009). Removal of the parathyroid glands will correct the set point for calcium and the patient will have lifelong normal or mild hypoalcaemia. As for CaR knockout models of NSHPT, homozygous knockout mice (-/- ) again exhibit a similar phenotype to NSHPT sufferers; that is, reduced growth and body weight, lethargy and decreased feeding, reduced bone mineral density and neonatal death (at ~day-14) (Ho et al, 1995). Specifically these CaR null mice exhibit high blood concentrations of ionised calcium, inappropriately high PTH secretion and relative hypocaliuria. Furthermore, their parathyroid glands are enlarged and exhibit chief cells hyperplasia (Ho et al, 1995).
1.5.2 Gain-of-function CaR mutations

Gain-of-function mutations cause the phenotype of autosomal dominant hypocalcaemia (ADH) (Hendy et al., 2000). The set-point of the receptor is left-shifted so it is more sensitive to calcium and inhibits PTH secretion inappropriately (Hendy et al., 2000). Sufferers may have mild-to-moderate hypocalcaemia (Pearce et al., 1996; Pollak et al., 1994). Like FHH, carriers may be asymptomatic depending on the severity of the mutation but may suffer from symptoms such as seizures or spasms (Hendy et al., 2000; Hendy et al., 2009). Treatment for hypocalcaemia generally involves use of calcium and vitamin D$_3$ supplementation, however this can cause complications in ADH patients such nephrolithiasis and nephrocalcinosis that can even lead to kidney failure (Hendy et al., 2009). Dozens of activating mutations have been reported in the literature (Hendy et al., 2000). They are clustered around amino acids 116 to 131 in the ECD where the receptor forms a dimer and also around the sixth and seventh TMD and third extracellular loop, which has been implicated in L-amino acid binding and transmission of conformational changes between the ECD and TMD (Hendy et al., 2009).

Bartter’s syndromes are a group of electrolyte imbalance diseases caused by a number of different receptors and channels (Thakker, 2004; Watanabe et al., 2002). Type V is caused by a gain-of-function mutation to the CaR (Thakker, 2004). The classical symptoms of Bartter’s syndrome are hypokalaemic alkalosis, hyperaldosteronism, hypereninaemia and also hypocalcaemia and hypercalciuria (Egbuna & Brown, 2008; Thakker, 2004). Type V Bartter’s is caused by a dominant gain-of-function mutation in the CaR (Vargas-Poussou et al., 2002; Watanabe et al., 2002); as previously discussed in the cTAL activation of CaR inhibits ROMK channels on the apical membrane. Constitutively active or overactive CaR would cause ROMK to become permanently inhibited. It is thought that the severity of the mutation causes Bartter’s syndrome (Watanabe et al., 2002).

1.5.3 Primary hyperparathyroidism

Parathyroid gland adenomas and FHH can be clinically hard to distinguish and diagnose (Hendy et al., 2009). Parathyroid adenomas cause excessive PTH secretion and hypercalcaemia however patients will have hypercalciuria. Patients may present with kidney stones due to chronic hypercalciuria, osteomalacia and be prone to fractures, muscle weakness and pain, fatigue, depression and anxiety (Coker et al,
Where nephrolithiasis is first diagnosed, persistent hypercalcaemia should be considered a possible warning sign of primary hyperparathyroidism (PHPT) (Coker et al, 2005). Full parathyroidectomy should correct the symptoms and have normal or mild hypocalcaemia. Future treatment to correct any osteomalacia or kidney function may be necessary. In rare cases PHPT can be caused by an ectopic adenoma secreting hormone anywhere in the body which can be difficult to find and remove.

1.5.4 Secondary hyperparathyroidism

Secondary hyperparathyroidism has a complex aetiology; degenerating kidney function causes hyperphosphataemia and hypocalcaemia [reviewed in (Felsenfeld et al, 2007; Rodriguez et al, 2005)]. Decreasing kidney function leads to the inability to excrete excess phosphate and reabsorb calcium and inability to produce 1,25-dihydroxyvitaminD₃ (Felsenfeld et al, 2007). In chronic kidney disease, the increased phosphate and lack of calcitriol leads to parathyroid gland hyperplasia. Current therapies for SHPT initially includes calcitriol, however this is less effective in end-stage renal disease, phosphate binders and the calcimimetic Cinacalcet are used (Rodriguez et al, 2005).

Chronic kidney disease is also associated with decreased bicarbonate reabsorption and which leads to acidosis. Senescence is also associated with secondary HPT and metabolic acidosis (Frassetto et al, 1996); possibly caused by declining renal function and failure to excrete excess phosphate and protons. It is possible therefore that increased PTH secretion via the pHₐ₅₆-sensitivity of the CaR might contribute to secondary HPT. Clinically, low bicarbonate concentration in pre-dialysis patients has been associated with increased coronary artery calcification score subsequently (Oka et al, 2012). Since oral sodium bicarbonate supplementation has been shown to slow the rate of decline of renal function in CKD patients with low plasma bicarbonate concentrations then the effect of this co-therapy on the development of secondary hyperparathyroidism and vascular calcification might also be considered (de Brito-Ashurst et al, 2009).

1.6 Calcium-sensing receptor signalling

The CaR exhibits pleiotropic actions via promiscuous coupling to multiple heterotrimeric G-protein subtypes including G₁₁₅, G₁₂/₁₃, G₁₅ and even G₁ in some breast and colon cancer cell lines (Ward, 2004; Ward & Riccardi, 2012). In this
section I will describe the variety of distinct signalling pathways induced by CaR activation.

![Figure 1.6. Schematic of CaR signalling.](image)

**Figure 1.6. Schematic of CaR signalling.** CaR activation induces $G_{i/o}$-mediated adenylate cyclase (AC) inhibition and decreased cAMP; $G_{12/13}$-mediated phospholipase D (PLD) activation and phosphatidic acid (PA) production; $G_{q/11}$ mediated activation of phospholipase C (PLC) which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to inositol trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ binds to IP$_3$ receptors (IP$_3$R) releasing intracellular calcium which activates protein kinase C (PKC) with DAG. Activation of the MAP kinases extracellular regulated kinase (ERK), c-jun amino-terminal kinase (JNK) and p38 kinase. Cytosolic phospholipase A2 (cPLA$_2$) activation leads to arachidonic acid (AA) production. Phosphatidylinositol 3 kinase (PI$_3$K) activation leads to PIP$_2$ formation.
1.6.1 Phospholipase activation

1.6.1.1 Phospholipase C

Before the CaR had been identified, a number of groups had already demonstrated PLC and PKC activation in bovine parathyroid cells in response to increasing extracellular calcium concentration (Brown et al., 1987; Brown et al., 1984; Hawkins et al., 1989; Lazarus et al.; Membreno et al., 1989; Nemeth et al., 1986; Shoback & McGhee, 1988). Furthermore, treatment with the PLC inhibitor U73122 has been shown to attenuate CaR-induced Ca\textsuperscript{2+} mobilisation and ERK phosphorylation (Godwin & Soltoff, 2002; Huang et al., 2002; Molostov et al., 2008; Remy et al., 2007; Ward et al., 2002). Finally the product of PLC-mediated inositol 4,5, bisphosphate (PIP\textsubscript{2}) hydrolysis, inositol trisphosphate (IP\textsubscript{3}), has been measured in CaR-expressing cells following exposure of the cells to CaR agonists (Chen et al.; Kifor et al., 1997) and other groups have used PI metabolism as a readout for CaR activity (Hu et al., 2000; Silve et al., 2005). Together, these studies indicate that CaR activation leads to the PLC-mediated degradation of PIP\textsubscript{2} into IP\textsubscript{3} and DAG. In chronic CaR activation, depletion of PIP\textsubscript{2} would result in attenuated signalling therefore it is significant that phosphoinositol 4-kinase (PI\textsubscript{4}K) is also activated following CaR activation as this will help replenish PIP\textsubscript{2} levels, thus permitting continued PLC activity (Huang et al., 2002). Significantly, the importance of CaR-mediated G\textsubscript{q/11} activation in the regulation of PTH secretion was best demonstrated by Wettschureck et al who showed that mice with a parathyroid-specific knockdown of G\textsubscript{q} on a G\textsubscript{11} null background develop severe primary hyperparathyroidism and hypercalcaemia (Wettschureck et al., 2007). Together, this establishes that CaR activation induces the G\textsubscript{q/11}/PLC pathway and this is of physiological importance for PTH secretion.

1.6.1.2 Phospholipases A2 and D

In CaR-HEK and bovine parathyroid cells, increasing Ca\textsuperscript{2+} concentration or addition of the CaR-activating aminoglycoside antibiotic neomycin, induces arachidonic acid (AA) release, effects which are susceptible to inhibition with general PLA\textsubscript{2} inhibitor arachidonyl trifluromethyl ketone (AACOCF\textsubscript{3}) (Handlogten et al., 2001; Kifor et al., 1997). Cytosolic PLA\textsubscript{2} activation is stimulated by PKC and can also be blocked by the PLC inhibitor U73122 (Handlogten et al., 2001; Kifor et al., 1997).
Activation of CaR expressed in MDCK cells results in PLD activation resulting from G12/13-mediated activation of the monomeric G protein Rho (Huang et al., 2004). Then in CaR-HEK cells, bovine parathyroid cells and MDCK cells, high extracellular calcium also stimulates the formation of phosphatidic acid (PA), which represents the principal product of PLD activity (Huang et al., 2004; Kifor et al., 1997). Furthermore, PKC has a stimulatory effect on PLD and increases its activity in acute treatments, whereas chronic treatment with PMA reduced CaR-induced PLD activity (Kifor et al., 1997).

1.6.2 Protein Kinase activation

1.6.2.1 Protein Kinase C

A number of groups demonstrated in the 1980s that PTH secretion could be increased in bovine parathyroid cells by the phorbol ester PMA, a known activator of PKC (Brown et al., 1984; Membreno et al., 1989; Nemeth et al., 1986). Furthermore, Ca\textsuperscript{2+} and Mg\textsuperscript{2+} evoked IP\textsubscript{3} formation and Ca\textsuperscript{2+} transients and increased in bovine parathyroid cells, which is indicative of PLC activation (Brown et al., 1987; Shoback et al., 1988).

In mGluR5, it has been proposed that the intracellular calcium oscillations this receptor elicited by the dynamic phosphorylation and dephosphorylation of an intracellular PKC site [reviewed in (Hermans & Challiss, 2001)]. Thus, as another class C GPCR with which this receptor shares homology, it is feasible that the CaR may share significant similarities in terms of its intracellular signalling.

CaR has five known putative PKC phosphorylation sites (Garrett et al., 1995). Work by Bai and co-workers demonstrated that mutation of Thr-646 and Ser-794 to alanine, did not alter CaR EC\textsubscript{50} for Ca\textsuperscript{2+} and thus are unlikely to be involved in any PKC-mediated regulation of the CaR (Bai et al., 1998b). In contrast, mutation of Ser-895 and Ser-915 both significantly reduced the EC\textsubscript{50} of Ca\textsuperscript{2+} for CaR however, the greatest reduction in EC\textsubscript{50} was observed when Thr-888 was mutated (Bai et al., 1998b). Importantly, an individual who had suffered lifelong hypocalcaemia was found to have a T888M mutation in their CaR (Lazarus et al., 2011). This gain-of-function mutation results in chronic suppression of their PTH secretion and thus has led to a diagnosis of ADH. The significance of this study is that it confirms in humans the importance of Thr-888 for the controlled inhibition of CaR activity in order to permit PTH secretion. It should be noted that I contributed to this study by
generating and validating the CaR^{T888M} mutant and then expressing it in HEK-293 cells for characterisation, including ERK activations assays, however this work will not be further described in this thesis (Lazarus et al, 2011).

Thus it would appear that phosphorylation of the PKC site CaR^{T888} (and possibly CaR^{S895} and CaR^{S915}) effectively switches CaR off by uncoupling from, or somehow inhibiting further, G_{q/11} / PLC activation. Furthermore, the extent of CaR^{T888} phosphorylation in the cell at any given moment is determined by relative changes in PKC and protein phosphatase (PP) activity [reviewed in (Ward & Riccardi, 2012)]. That is, the PP1/PP2A inhibitor calyculin-A prevents dephosphorylation of CaR^{T888} causing suppression of CaR-induced Ca^{2+} mobilisation (Davies et al, 2007). Since the PP1 inhibitor tautomycin is without effect, our working hypothesis is that PP2A is the phosphatase responsible for CaR^{T888} dephosphorylation although this has not yet been confirmed using siRNA knockdown. That said however, the catalytic subunit of PP2A was found by dual-antibody immunofluorescence to colocalise with pCaR^{T888} immunoreactivity (Davies et al, 2007). Significantly, I was further able to demonstrate that CaR activation induces PP2A activation in CaR-HEK cells, again supporting the hypothesis that PP2A could be the CaR^{T888} phosphatase (McCormick et al, 2010). Again though, this work will not be further described in this thesis. The physiological significance of this is that in human PT cells, our collaborator Professor Conigrave (University of Sydney) found that calyculin-A permitted PTH secretion even under high Ca^{2+} o conditions apparently by preventing CaR^{T888} dephosphorylation and thus suspending CaR’s suppressive action on secretion (McCormick et al, 2010).

1.6.2.2 Protein Kinase A

There is little direct evidence of a role for PKA in regulation of CaR. However, Bosel et al found that use of the PKA inhibitor H89 modestly increased CaR-induced PI hydrolysis, this effect being additive to the effect of inhibiting PKC simultaneously; suggesting that the two pathways might converge (Bosel et al, 2003). Then in the pituitary cell-line AtT-20, the CaR drives PTHrP and ACTH secretion, effects that are inhibited by H89, however AtT-20 cells represent one of the unusual instances whereby CaR actually couples to G_{alpha} to stimulate cAMP generation (Mamillapalli & Wysolmerski).
With regards the possible interaction between cAMP and CaR signalling, Gerbino et al reported that increasing intracellular cAMP levels while the CaR is mediating Ca\textsuperscript{2+} oscillations, causes an increase in the frequency of those oscillations. (Gerbino et al, 2005). However, whether the cAMP was acting at the level of the IP\textsubscript{3}R or the CaR itself was not clarified and there have been no further reports on this topic to date.

In fact, the human CaR contains two putative sites for PKA phosphorylation, at Ser-899 and Ser-900 (Garrett et al, 1995) and thus one might speculate that the cAMP could be affecting the CaR itself, perhaps via PKA-mediated phosphorylation. Therefore, here the functional roles of pseudo-phosphorylated and phosphor-null Ser-899 and Ser-900 mutants were investigated in the current study. The role of Ser-899 phosphorylation in the binding of 14-3-3 proteins (section 1.6.4.4) has been investigated by Breitweiser and co-workers (Grant et al, 2011; Stepanchick et al, 2010) however, to date there are no reports on the consequence of mutating either CaR\textsuperscript{S899} or CaR\textsuperscript{S900} on receptor signalling.

1.6.3 Mitogen-Activated Protein Kinases

The mitogen-activated proteins kinases (MAPKs) are a family of protein kinases that are activated by dual phosphorylation on neighbouring tyrosine, serine/threonine residues. They are sometimes referred to as proline-directed kinases as their consensus sequences include a proline upstream of the serine/threonine target site. They include the extracellular signal-regulated kinase (ERK), c-Jun N-Kinases (JNKs) and p38 kinases (Ward, 2004).

1.6.3.1 Extracellular Regulated Protein Kinase

In various CaR-expressing cell models, agonists including Ca\textsuperscript{2+}, Gd\textsuperscript{3+}, neomycin and spermine all increased ERK phosphorylation (Kifor et al, 2001; Ward et al, 2002; Yamaguchi et al, 2000). In parathyroid cells, CaR-HEK cells and opossum kidney (OK) cells ERK phosphorylation was found to be downstream of G\textsubscript{i}, PLC, and PKC (Kifor et al, 2001; Ward et al, 2002). Furthermore, Hobson et al reported that in CaR-HEK cells, CaR-induced ERK activation can be inhibited by the PI3K inhibitors, wortmannin and LY294002 (Hobson et al, 2003). In human parathyroid cells ERK
is directly phosphorylated by mitogen-activated kinase (MEK); inhibition of MEK1 causes an apparent attenuation of PTH secretion (Corbetta et al, 2002).

In bovine and human parathyroid cells, CaR is associated with membrane caveolae (Kifor et al, 1998) and activation of CaR with high Ca$^{2+}$ increased the phosphorylation of ERK in the caveolae (Kifor et al, 2003). Indeed, disturbance of the caveolae caused disruption of CaR-mediated ERK phosphorylation in bovine PT cells (Kifor et al, 2003).

1.6.3.2 p38 MAP kinase

In CaR-HEK cells, high Ca$^{2+}$ stimulates p38 kinase phosphorylation; likewise in the CaR expressing cell-lines H-500 Leydig cells and MCETE-E1 mouse osteoblast cells, CaR agonists induce p38 kinase phosphorylation (Tfelt-Hansen et al, 2003; Yamaguchi et al, 2000). In CaR-HEK cells inhibition of p38 decreased PTHrP secretion and Leydig H-500 cells also decreased proliferation (MacLeod et al, 2003).

1.6.3.3 C-Jun N-terminal Kinase

High Ca$^{2+}$ and Gd$^{3+}$ induce JNK phosphorylation (Arthur et al, 2000; Chattopadhyay et al, 2004). Like the other MAPKs ERK and p38 kinase, PTHrP secretion is sensitive to the JNK inhibition in Leydig H-500 cells (Tfelt-Hansen et al, 2003). Furthermore, in calvarial osteoblastic cells, CaR agonists increased JNK phosphorylation while JNK inhibition using SP-60012500125, significantly decreased proliferation (Chattopadhyay et al, 2004). Arthur et al have shown that in MDCK cells, high Ca$^{2+}$-induced JNK activation is decreased by inhibition of $G_i$ with pertussis toxin (PTx) (Arthur et al, 2000).

1.6.4 Calcium-sensing receptor-interacting proteins

1.6.4.1 Beta arrestin

β-arrestins are involved in GPCR desensitisation and internalisation (Lorenz et al, 2007; Pi et al, 2005). Metabotropic GluRs are known to bind β-arrestin and causing the receptors to causes desensitise and internalise: given the high level of homology between class C receptors it is likely they also bind β-arrestin (Mundell et al, 2001).
One unique factor about CaR is their low level of desensitisation given that it is constitutively stimulated. Using RT-PCR, β-arrestin1 and 2 was found in human parathyroid glands (Pi et al, 2005) and we also find β-arrestin1 in our bovine microarray (table 4.1). Measuring CaR activity in HEK-293 cells with a luciferase reporter showed that co-transfection with either β-arrestin 1 or 2 caused a significant decrease in activity (Pi et al, 2005). β-arrestin KO mice had significantly decreased basal levels of PTH and PTH was reduced in response to hypocalcaemia, interestingly though they had normal blood calcium levels indicating a level of compensation (Pi et al, 2005).

Lorenz et al showed that co-expression of the CaR and β-arrestin-1/-2 significantly decreased IP formation in response to Ca\(^{2+}\), whereas using siRNA to reduce β-arrestin increased IP formation (Lorenz et al, 2007). Mutation of the 5 PKC phosphorylation sites on the CaR, or inhibition of PKC with GFX, rendered the receptor more sensitive to CaR agonists, and abolished the β-arrestin sensitivity (Lorenz et al, 2007).

1.6.4.2 Calmodulin

The mGluR7a c-terminus has also shown binding capabilities to calmodulin where it competes for binding to G-protein βγ (O'Connor et al, 1999). Rey et al showed that CaR-mediated oscillations induced by L-Phe could be potentiated by calmodulin inhibitors (Rey et al, 2006). The same group suggested that this could be indicative of agonist dependent signalling however, we are not aware of the effects of calmodulin inhibitors other CaR agonists (Rey et al, 2006).

Using a calmodulin target database Huang et al predicted a calmodulin binding site on CaR between amino acids Phe-881 and Val-894, an area which shares homology with the mGluR7a binding region (Huang et al). Mutation at positions 1,8 and 14 in the motif to phosphomimetic amino acids rendered the CaR less sensitive with resultant increases in EC\(_{50}\)s for Ca\(^{2+}\) (Huang et al, 2010). Mutation also caused an increase in agonist-stimulated internalisation, suggesting a decrease in receptor stability at the membrane. Interestingly, the calmodulin inhibitor W-7 caused an increase in receptor sensitivity and maximum response, inferring that calmodulin binding is negatively regulating the CaR (Huang et al, 2010).
1.6.4.3 Potassium channels

Yeast two hybrid screening showed that the CaR C-terminal interacts with the inwardly rectifying potassium channel Kir4.1 (Huang et al, 2007a). Expression in HEK-293 cells showed that WT but not dominant negative CaR could co-immunoprecipitate with Kir4.1 and Kir4.2, and immunohistochemistry in rat nephrons and HEK-293 cells showed that they co-localised in the DCT (Huang et al, 2007a). Expression in Xenopus laevis oocytes showed that activation of WT CaR with Gd\(^{3+}\) inhibited Kir4.1 and Kir4.2, and channel and receptors colocalised (Huang et al, 2007a) The same group went on to shows that CaR reduces Kir4.1 activity by reducing cell surface expression (Cha et al, 2011). The CaR, Kir4.1 and caveolin-1 coimmunoprecipitated from HEK-293 and rat kidney cortex cells, and using siRNA to reduce caveolin-1 reversed the inhibition by the CaR (Cha et al, 2011).

1.6.4.4 14-3-3 proteins

14-3-3 proteins are adapters or chaperones that are ubiquitously expressed; they have seven isoforms and have been linked to a range of signalling pathways and endoplasmic reticulum (ER) retention (Arulpragasam et al, 2012; Breitwieser; Grant et al, 2011; Stepanchick et al, 2010). Two yeast linked hybridisation showed that CaR C-terminal can interact with two 14-3-3 proteins theta (θ) and zeta (ζ). Over expression of either in CaR-HEK cells decreased Rho-mediated SRE activation but not ERK activation (Arulpragasam et al, 2012). This interaction was PKC independent and the 14-3-3 consensus motif on the ICD is not required for 14-3-3 θ interaction (Arulpragasam et al, 2012).

Breitwieser et al hypothesise that 14-3-3 proteins mediate CaR ER retention, via Serine-899 (Grant et al, 2011). Co-immunoprecipitation experiments show that mutation of Ser-899 to alanine, increased interaction causing, whereas mutation to the phosphomimetic amino acid aspartate, caused decreased interaction and increased plasma membrane expression (Grant et al, 2011). The functional importance of Ser-899 will be dealt with in chapter 4.
1.6.4.5 Other interactions

Filamin is a matrix protein that binds actin, but has also been shown to mediate intracellular signalling. Yeast two-hybrid analysis and co-immunoprecipitation experiments have shown that CaR and filamin-A directly associate (Awata et al., 2001; Pi et al., 2002). Filamin-A and CaR colocalise in parathyroid cells and CaR-HEK cells (Awata et al., 2001; Hjalm et al., 2001). By using a dominant negative filamin-A Hjalm et al also showed that filamin-A facilitates ERK phosphorylation and disruption decrease activation (Hjalm et al., 2001). Interestingly, Rey et al have shown that disruption of filamin-A, G12, Rho or actin disrupt L-Phe signalling; inferring a possibly mechanism for ligand specific signalling (Rey et al., 2005). In CaR-HEK cells filamin was immunoprecipitated with CaR and also Rho; disruption of filamin-A binding inhibited Rho mediated SRE activation (Pi et al., 2002).

Non-selective cation channels transport Na\(^+\), K\(^+\) and Ca\(^{2+}\) and are present in hippocampal neurons and HEK cells (Ye et al., 1996a; Ye et al., 1996b). Exposing these cells to CaR agonists, Ca\(^{2+}\), neomycin and spermine, increased the open probability of non-selective cation channels in CaR-HEK cells and rat hippocampal neuronal cells (Ye et al., 1996a; Ye et al., 1996b). CaR activation is also linked to transient receptor potential canonical 1 (TRPC1) and voltage gated L-type calcium channels (El Hiani et al., 2009a; El Hiani et al., 2006; El Hiani et al., 2009b; Parkash, 2008a; Parkash, 2008b; Topala et al., 2009).

1.7 Summary

It has been known for nearly a decade that the CaR is sensitive to large changes in pH\(_o\). In addition, experiments in dogs have revealed that metabolic acidosis or alkalosis directly potentiate or inhibit PTH secretion respectively. Work by this laboratory has shown that the CaR itself is sensitive to pathophysiological by relevant changes in pH\(_o\) and can significantly alter PTH secretion in an \textit{ex vivo} model. However in \textit{vivo}, calcium and proton buffers (e.g. albumin) could potentially compensate for decreasing blood pH by releasing calcium. Thus, it is necessary to determine whether CaR retains its pH\(_o\) sensitivity even in the presence of albumin. Furthermore, the extracellular residue(s) responsible for mediating CaR pH\(_o\) sensitivity have not been identified.

Regarding cAMP and the CaR, there is evidence that increasing intracellular cAMP concentration drives PTH secretion and thus one-way to stop excess secretion of
PTH would be if cAMP also activated the CaR thus inhibiting further PTH secretion. This would allow rapid, fine-control of PTH secretion. Here feedback of cAMP on the CaR is examined by studying changes in Ca^{2+} mobilisation following manipulations of cAMP levels or of putative PKA sites in the CaR ICD. Thus, both of the CaR’s two putative PKA phosphorylation sites were mutated in order to determine these residues’ influence, if any, on CaR signalling and cAMP responsiveness.

1.8 Aims and objectives

The overall aim of the project was to further understand the molecular mechanisms underlying the control of extracellular calcium homeostasis, with particular regards to the action and signalling of the calcium-sensing receptor.

The specific objectives of the project were:

Objective 1: To determine the extracellular residue(s) responsible for CaR pHs sensitivity. This undertaking included the mutation of the sixteen extracellular histidine residues and single free-cysteine residue sequentially, followed by transient expression in a heterologous expression system namely HEK-293 cells (see Chapter 3).

Objective 2: To investigate whether chronic acidosis or alkalosis could be compensated for by changes in the buffering of calcium ions (see Chapter 3).

Objective 3: To determine the effect of increasing intracellular cAMP concentrations on calcium-sensing receptor activation, with particular regard to the effects of cAMP on determining Ca^{2+}_o threshold concentrations (see Chapter 4).

Objective 4: To investigate the effect of mutation of the two putative PKA sites in the CaR carboxyl terminus on receptor function (see Chapter 4).
Chapter 2

Materials and Methods
2.1 Cell culture

Wild-type HEK-293 cells were grown in Dulbeccos’ modified Eagle’s medium fortified with 10% foetal bovine serum (FBS) and split twice weekly by trypsinisation. HEK-293 cells stably transfected with human CaR were maintained in DMEM, 10% FBS with 1mg/ml Hygromycin B for selectivity. Cells were kept in an incubator at 37°C with 5% CO₂ and not maintained beyond passage 35.

2.2 Bovine parathyroid cell isolation and culture

Bovine parathyroid glands were isolated from recently slaughtered cattle which were under thirty months and placed in collection buffer containing 141 mM NaCl, 5.3 mM KCl, 20 mM HEPES, 5 mM MgSO₄ and 2 mM CaCl₂ (pH 7.4). The glands were chilled during transportation. The glands had any excess fatty tissue removed and then injected and finely minced in calcium buffer containing 20 mM HEPES, 125 mM NaCl, 4 mM KCl, 0.5 mM MgCl₂, 5.5 mM glucose and 0.5 mM CaCl₂ supplemented with 75µg/ml DNase I and 475U/ml collagenase type I. The minced parathyroid gland in the collagenase solution was shaken at 180 rpm for 20 min at 37°C, then triturated and filtered using a 30 µM monofilament filter cloth. This was repeated with any undigested tissue. The filtrate was centrifuged at 100rpm for 5 min at room temperature and the supernatant discarded. The pellet was resuspended in a 30% Percoll solution [30% (v/v) Percoll, 70% (v/v) 1.4 X collection buffer] and centrifuged at 18800g for 30 min at 15°C. Excluding the bottom 1ml, the supernatant was removed and the pellet resuspended along with the remaining supernatant and cultured on to 13 mm glass coverslips coated with 0.01% (v/v) rat tail collagen type I in PBS. The bovine parathyroid cells were cultured in low-glucose (5.56 mM) DMEM supplemented with 10% heat-inactivated FBS and (0.6U:0.6ug/ml) penicillin-streptomycin, at 37°C with 5% CO₂ and cultured for no more than 3 days.

2.3 Transient cell transfection

Transient transfections were performed using either FuGene6 or TransIT-LT1 reagents according to manufacturers’ instructions using approximately 60% confluent cells. Briefly, 3µl transfection reagent was added to serum-free DMEM and incubated at room temperature for 5 minutes. 1µg DNA vector was then added
left for a further 15-30 minutes. The solution was then added to the cells drop-wise. Cells were used experimentally within 72 hours of transfection.

2.4 Preparation of cell lysates

Cells were lysed using an appropriate volume of RIPA buffer; 12 mM HEPES (pH 7.6), 300 mM mannitol, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton-X 100, 0.1% (w/v) sodium dodecyl sulphate (SDS), 1 mM NaF, 250 µM sodium pyrophosphate, 100 µM sodium vanadate, 1.25 µM pepstatin, 4 µM leupeptin, 4.8 µM PMSF and 1 mM N-ethylmaleimide (NEM). Cells were first washed with ice-cold 1x PBS and incubated with RIPA buffer for 15 minutes before collection. All cell lysates were stored at -80°C.

2.5 Immunoblotting

Cell lysates were diluted with 5X Laemmli buffer containing 320 mM Tris base (pH 6.8), 5% (w/v) sodium dodecyl sulphate, 25% (v/v) glycerol, 1% (w/v) bromophenol blue in the presence of 5% (v/v) β-mercaptoethanol reducing agent in a 1:4 ratio and incubated at 85°C (65°C for CaR blots) for 3 mins. Samples were separated using SDS-polyacrylamide gels in running buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS.

Proteins were transferred electrophoretically on to 0.45 µM nitrocellulose transfer membrane for 1 hour at 200 milliAmps in blotting buffer; 25 mM Tris, 200 mM glycine, 15% (v/v) methanol. The non-specific binding sites on the membrane were blocked using Tween-TBS buffer (15 mM Tris (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween 20) with 1% bovine serum albumin or 2% skimmed milk for (30 minutes at room temperature). Following this step the membrane was probed with primary antibody in Tween-TBS (1 hour at room temperature). Before probing the membrane with secondary antibody the membrane was washed with Tween-TBS (20 minutes). The horseradish peroxidase conjugated secondary antibody was either anti-mouse or anti-rabbit (1:5000 dilution) and incubated for 1 hour. Finally the membranes were washed Tween-TBS (20mins) and developed using ECL chemiluminescence reagents as per the manufacturer’s instructions. Densitometry was performed for semi-quantitative analysis of the immunoreactive bands.
2.6 Intracellular calcium imaging

Dual-excitation wavelength microfluorometry was carried out on cells cultured on 13 mm glass coverslips for between 24-48 hours in DMEM with 10% FBS. They were loaded with 1 µM FURA2-AM in 1.2 mM Ca²⁺ experimental buffer supplemented with 0.1% (w/v) BSA for 1-2 hours (room temperature in the dark). Experimental buffer contained in detail; 0.5-5.0 mM CaCl₂, 20 mM HEPES, 118-125 mM NaCl, 0.5 mM MgCl₂, 4 mM KCl and 5.5 mM glucose (warmed to between 25-32°C). The pH was adjusted using NaOH and HCl solutions. The cells were mounted in a perfusion chamber and observed through a x40 oil-immersion lens on a Nikon Diaphot inverted microscope. Baseline conditions for HEK293 were measured in 0.5 mM CaCl₂ and 0.8 mM CaCl₂ for bovine parathyroid gland (bPTG) cells. Images were collected using Metafluor Software and quantified using GraphPad Prism software.

2.7 Site-Directed Mutagenesis

QuickChange® Lightning Site-directed mutagenesis of WT human CaR was undertaken in accordance with the manufacturers’ instructions (Stratagene). Primers were ordered from Sigma-Aldrich (UK). Briefly, 100 ng WT CaR plasmid was mixed with 50 ng forward and reverse mutant primers, buffer and enzyme.

The mix was placed in a thermal cycler and subjected to the temperature changes summarised in table 2. Following this the WT DNA was digested with DPN1 restriction enzyme for 5 minutes at 37°C. The mutant plasmid DNA was then transformed in to XL10-Gold® cells in accordance with manufacturers’ instructions. Briefly 2 µl of mutant DNA was added to 40 µl of XL10-Gold cells and chilled on ice for 30 minutes. Cells were then heat shocked for 40 seconds in a 42°C water-bath before being placed on ice for 2 minutes. 500 µl of warmed NZY broth was added and then solution shaken at 37°C for 1 hour. The transformed bacteria were spread on agar plates containing ampicillin (100 µg/ml) and left overnight at 37°C. Two colonies were then picked and grown in 3ml LB-broth for 8 hours and then transferred to 50ml LB broth containing ampicillin (200ug/ml) and left overnight at 37°C shaking. The mutation was confirmed by sequencing the DNA using the University of Manchester in-house service.
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Table 2. Summary of thermal cycler protocol. To perform site-directed mutagenesis, template wild-type plasmid, mutation-containing primers, dNTPs, PCR buffer and enzyme were subjected to heating and cooling cycles to mutate the human CaR.

2.7.1 Mutation primers
Below are the primers used to generate the site-directed mutagenesis of human WT CaR.

His41Val
Forward: 5’-CTTGGGGGGCTCTTTTCTATTGTTTTTGAGTAGCAGCTAAAG-3’
Reverse: 5’-CTTTAGCTGCTACTCCAAAAACATAGGAAAGAGCCCCCAAG-3’

His134Val
Forward: 5’-TTCTGCAACTGCTCAGAGGTCATTCCCTCTACGATTGC-3’
Reverse: 5’-GCAATCGTAGAGGGAATGACCTCTGTAGCAGTTGCAGAA-3’

His192Val
Forward: 5’-CATCCCCAATGATGAGGTCCAGGCCACTGCCATG-3’
Reverse: 5’-CATGGCAGTGGCCTGGACCTCATCATGGGGATG-3’

His254Val
Forward: 5’-TGATGAGGAAGAGATCCAGGAGGGCAGCGGACTGCTCCA-3’
Reverse: 5’-TTGATCACCTCTCACCACAACTGGATCTCTCTTCATCA-3’

His312Val
Forward: 5’-ATGCCTCAGTACTTCGTCGTTGTTGCGGGGCAC-3’
Reverse: 5’-GTGCCGCAACCACGAGCAGGTACTGAGGAT-3’
His338Val  
Forward: 5'-GAAATTCCTGAAGAAGGTCGTTCCAGGAAGTCTGTCCA-3'  
Reverse: 5'-TGGACAGACCTTCTGGGAACGACCTTCTCCAGGAATTC-3'

His344Val  
Forward: 5'-GAAATTCCTGAAGAAGGTCGTTCCAGGAAGTCTGTCCA-3'  
Reverse: 5'-TGGACAGACCTTCTGGGAACGACCTTCTCCAGGAATTC-3'

His359Val  
Forward: 5'-GGGAAGAAACATTTAAGTTATCCTGAAGAAGGTCGTTCCAGGAAGTCTGTCCA-3'  
Reverse: 5'-CTTTTGACCTTTTCTGGGAACGACCTTCTCCAGGAATTC-3'

His377Val  
Forward: 5'-GGCACACCTTTTCTGGGAACGACCTTCTCCAGGAATTC-3'  
Reverse: 5'-GTGGACCCACCTTTCTGGGAACGACCTTCTCCAGGAATTC-3'

His413Val  
Forward: 5'-GACCCCTTACATAGATTACACGGTTTTACGGATATCCTACAATGTG-3'  
Reverse: 5'-CACATTGTAGGATATCCGTTAAAACCCGTGAATCTATGTAAGGGGTC-3'

His429Val  
Forward: 5'-AGCAGTCTACTCCATTTCSGCGTGCCTTGCAAGAAGATATATATC-3'  
Reverse: 5'-ATATATATATCTTGCAAGGCGACCGGAATGGAGTAGACTGGTCC-3'

His463Val  
Forward: 5'-CGTGCCAGGTTCTTAAGGTCTTGCCTACGGCATCTAAAC-3'  
Reverse: 5'-GTATGAGTCCGCTAGGACCTTAAAGGACCTTCCAGGACCTG-3'

His466Val  
Forward: 5'-CAGGTCTCTTAAGGTCTTGAAGAGTTTACGTTGGAATCAACATATGCTGAAGAGTTTACGTTGGAATCAACATATG-3'  
Reverse: 5'-CATATTGTTTGTAAAGGTCAAACGTAGGACCTTAAAGGACCTG-3'
His495Val
Forward: 5’-ATTCATCATAACTGGGTCTCTCTCCCCAGAGGATG-3’
Reverse: 5’-CATCCTCTGGGAGAGGACCCAGTTGATGATGGAAT-3’

His595Val
Forward: 5’-CTTCTGGTGCAATGAGAACGTCACCTCCTGCATTGCAAG-3’
Reverse: 5’-CTTGGCAATGCGAGGGTACGTTCTCATGGACCAGAAG-3’

His766Val
Forward: 5’-CATCTTCATACGTGCGTCGAGGGCTCCCTCATGG-3’
Reverse: 5’-CCATGAGGGACCCCTCGACGACGTGATGAAGATG-3’

Ser899Ala
Forward: 5’-TCCCGCAAGCGGGCCAGCCTTG-3’
Reverse: 5’-CAAGGCTGCTGGCCCGCCTTG-3’

Ser899Asp
Forward: 5’-CTTCGGCAAGCGGGACAGCCTGGAGG-3’
Reverse: 5’-CTTCAAAGGCTGTGCTGCCGGAGGA-3’

Ser900Ala
Forward: 5’-CGCAAGCGGTCCGAGGCTGGAGG-3’
Reverse: 5’-GCTTCAAGGCTGGCGACCGAGTCG-3’

Ser900Asp
Forward: 5’-CGCAAGCGGTCCGAGGCTGGAGG-3’
Reverse: 5’-GCTTCAAGGCTGGCGACCGAGTCG-3’

2.7.2 Sequencing primers
Below are the primers used to sequence the CaR mutants generated using the primers in section 2.7.1.

His41Val
Forward 365 5’ GCAGAACATGGCATTTATAG 3’
His681Val and His192Val
Forward 681 5’ CGTTTCTAAGGCCCTTGAAG 3’

His254Val and His312Val
Forward 1001 5’ GCACAATTGCAGCTGATGAC 3’

His338Val and His344Val
Forward 1322 5’ CCATTGGATTGCTCTGAAG 3’

His359Val and His377Val
Forward 1383 5’ CCATCCCAGGAAGTCTGTCCA 3’

His413Val and His429Val
Forward 1509 5’ AAAGTGGCCACAGGGTCTGC 3’

His463Val and His466Val
Forward 1642 5’ GTCTACTCCATTGCCACCAG 3’

His496Val
Forward 1781 5’ CAAACAATATGGGGGAGCAG 3’

His595Val
Forward 1963 5’ AGTGGGTTCTCCAGGGAGCAG 3’

His766Val
Forward 2212 5’-GATCGACTCACCTCTTTG-3’

Ser899Ala, Ser899Asp, Ser900Ala, Ser900Asp, Ser899/900Ala, Ser899Asp/Ser900Asp
Forward 2906 5’-CATCGAGAGGAGGTGGCGTTG-3’

2.8 Microarray

Total RNA was extracted from bovine parathyroid glands using a RNeasy Lipid Tissue Midi Kit (Qiagen, UK) according to manufacturer’s protocol. Previous to extraction the glands had been kept in RNAlater solution (Qiagen) at room temperature. The RNA was run on an agarose gel to certify quality. Microarray was
carried out using GeneChip Bovine Genome Array (Affymetrix. High Wycombe, UK) at the University of Manchester in-house service.

2.9 ERK Assay

CaR-HEK cells were grown on 30 mm dishes in DMEM fortified to 10% FBS at 37°C in 5% CO₂, for 24-48 hours. The dishes were placed on heat blocks at 37°C in 0.5 mM calcium buffer for 20 minutes to equilibrate. They were then subjected to treatment in appropriate buffer for 5 minutes before being washed in ice cold PBS. Care was taken to ensure cells did not wash away. 150 µl RIPA buffer was then added to the dish, which was kept on ice for 15 minutes. The cellular debris and buffer was collected and centrifuged at 15000 RPM for 10 minute. Samples were stored at -80°C, until they were run on immunoblotted.

2.10 Protein references

Proteins used for comparative analysis were taken from GenBank® part of the NIH genetic sequence databank. GenBank® or NCBI references are as follows; Human CaR AAI12237.1, Bovine CaR NP_776427, Mouse AAD28371.1, Rat AAO59490.1, Dog ABD16381.1 and Cat NP_001158126.1. Metabotropic glutamate receptor 1 AAI36281.1 metabotropic glutamate receptor 4 isoform 1 NP_000832.1 metabotropic glutamate receptor 5 splice variant d AAT37960.1, metabotropic glutamate receptor 8 isoform b NP_001120795.1

2.11 Statistical analysis

N refers to the number of coverslips used per experiment or, in the case of immunoblot experiments N refers to the number of 30 mm dishes collected per variable. At least 2 cells, average 10 cells, were visualised per coverslip. Data were analysed using GraphPad Prism (V5) and Microsoft excel. In order to determine the change in Fura2 ratio the area under the curve (trace line) were quantified using GraphPad Prism software. For experimental reasons the first minute of each experiment was excluded. Data is presented ± S.E.M and P values <0.05 were considered significantly different.
Chapter 3

Modulation of calcium-sensing receptor activity by extracellular pH
3.1 Introduction

In light of work by Quinn et al and more recent unpublished research in the current laboratory, it is clear that the CaR is sensitive to small changes in pH<sub>o</sub> in vitro (Quinn et al, 2004). However, two significant issues remain unresolved. The first is whether the abundance of albumin in the blood may counteract the acid-sensitivity of the CaR given that it can increase its Ca<sup>2+</sup>-binding capacity under alkaline conditions and liberates Ca<sup>2+</sup> under acid conditions. Therefore, it is necessary to determine whether the sensitivity of the CaR to pathophysiological changes in pH<sub>o</sub> is still observed in the presence of albumin at close to its physiological concentration in plasma. Then, another outstanding issue regards the identification of the amino acid residue(s) responsible for CaR pH<sub>o</sub> sensitivity which otherwise remains unknown. With extracellular histidine and (free) cysteine residues the amino acids whose sidechain pK values are closest to the physiological pH (7.35-7.4), these are arguably the most likely to confer pH<sub>o</sub> sensitivity on the CaR. As a result, I mutated all 16 extracellular histidines and 1 free-cysteine to neutral residues to determine whether a reduction or loss of pH<sub>o</sub>-sensitivity results.

3.2 Materials and Methods

CaR-HEK cells were cultured as described in (Chapter 2) section 2.1. Bovine PTG cells were isolated and cultured as detailed in section 2.2. Intracellular calcium imaging was performed as described in sections 2.6. Site-directed mutagenesis was performed under the conditions described in section 2.7, using the primers from section 2.7. The receptor was sequenced in the mutated region to confirm identity of the mutant.

3.3 Effect of albumin addition on extracellular pH sensitivity in CaR-HEK cells and bovine parathyroid cells

Less than half of circulating ionised calcium is free ionised and thus able to be detected by the CaR in vivo. Of the remainder, the majority is bound to blood proteins such as albumin which has a blood concentration of 35-50g/L (i.e. 3.5-5% w/v) (Kragh-Hansen & Vorum, 1993). The binding of calcium to albumin in the blood is sensitive to blood pH such that under acidic conditions calcium is displaced from the albumin whereas under alkaline conditions more calcium binds. Therefore,
this may counteract the idea that $\text{pH}_o$ affects PTH secretion. That is, under acidic conditions CaR sensitivity to $\text{Ca}^{2+}_o$ falls however the amount of free ionised $\text{Ca}^{2+}$ rises due to its release from albumin. Then under alkaline conditions, CaR sensitivity to $\text{Ca}^{2+}_o$ rises however with less free ionised $\text{Ca}^{2+}$ available due to its binding to albumin.

Thus, I examined this possibility by studying CaR-induced $\text{Ca}^{2+}_i$ mobilisation in bovine parathyroid cells in the presence of 5% (w/v) bovine serum albumin (BSA) and during phases of elevated and lowered $\text{pH}_o$. Exposing cells to 2.5 mM $\text{Ca}^{2+}_o$ in the presence of 5% (w/v) BSA, induced $\text{Ca}^{2+}_i$ oscillations that were significantly attenuated when $\text{pH}_o$ decreased from 7.4 to 7.2 (**P<0.001 by paired t-test, N≥4; Figure 3.1). Similarly, increasing $\text{pH}_o$ from 7.4 to 7.6 significantly increased the response despite the presence of BSA (*P<0.05 by paired t-test, N≥4; Figure 3.1). Therefore, the presence of albumin was insufficient to prevent changes in $\text{pH}_o$ from altering $\text{Ca}^{2+}_o$ sensitivity in bovine parathyroid cells as before.
Figure 3.1. Effect of pH₀ on bovine parathyroid cells in the presence of 5% (w/v) albumin. Panel Ai) Representative trace showing changes in Ca²⁺ᵢ (Fura2 ratio, 350/380 nm) in 2 single cells (orange and purple) and in a “global” cluster of cells (black), in response to 2.5 mM Ca²⁺₀ and following a temporary increase in pH₀ from 7.4 to 7.6. Quantification of the change in area under the curve/min (calculated as described in chapter 2 Materials & Methods) is shown as a bar graph in Aii. Panel B) As for panel A except with a decrease in pH₀ instead, from 7.4 to 7.2. *P<0.05, ***P<0.001 by paired t-test, N≥4.

In order to directly ascribe the pH₀ sensitivity to the CaR itself as opposed to another target on the parathyroid cell membrane, these experiments were then repeated in CaR-HEK cells. Again it was found that the presence of 5% BSA failed to prevent the pH₀-dependent change in CaR sensitivity. Lowering pH₀ by 0.2 units significantly decreased Ca²⁺ᵢ mobilisation, while increasing pH₀ by 0.2 units significantly increased Ca²⁺ᵢ mobilisation as before (*P<0.05 by paired t-test, N≥6; Figure 3.2).
Figure 3.2. Effect of pH\textsubscript{o} on CaR-HEK in the presence of 5\% (w/v) albumin. Panel A) representative trace showing changes in Ca\textsuperscript{2+}\textsubscript{i} (Fura2 ratio, 350/380 nm) in 2 single cells (orange and purple) and in a “global” cluster of cells (black), in response to 3 mM Ca\textsuperscript{2+}\textsubscript{o} and following a temporary increase in pH\textsubscript{o} from 7.4 to 7.6 (i). Quantification of the change in area under the curve/min is shown as a bar graph (ii). Panel B) As for panel A except with a decrease in pH\textsubscript{o} from 7.4 to 7.2. *P<0.05 by paired t-test, N≥6.
3.4 Determination of the contribution of extracellular histidine residues to calcium-sensing receptor \( \text{pH}_o \)-sensitivity

### 3.4.1 Confirmation of each CaR histidine mutant’s base sequence and protein expression in HEK-293 cells.

In the ECD and extracellular loops of the human CaR, 16 histidine residues were identified and mutated to valine by Lightning QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies Inc. CA. USA) as described in detail in Section 2.7. All residues were mutated individually, with the exception of His-466 which was co-mutated with His-463 due to the close proximity of the residues. Successful introduction of each mutation to the CaR vector was confirmed by sequencing as described in Section 2.7 and is shown in each Panel A of Figures 3.3-3.16. These panels also show a 20 amino acid portion of the resulting predicted amino acid sequence. It was common to receive wild-type sequence back showing that the mutagenesis had not been successful and thus in a substantial number of cases alternate colonies were selected, or, the sequencing reaction was repeated and new colonies selected.

Next, in order to confirm that each CaR mutant protein could actually be expressed, each mutant was transiently transfected, with either Miras (Geneflow) or Fugene6 (Promega) detergents in a 3:1 ratio of reagent (µl) to DNA (µg), into HEK-293 cells with the cells then lysed using modified RIPA buffer after 48 hours. The lysates were resolved by SDS-PAGE and the membranes probed with either anti-CaR monoclonal antibody (ADD), or, with anti-β-actin antibody as a loading control. The β-actin loading controls for all of the mutants were similar to that of WT, indicating that protein loadings were equivalent throughout (Figures 3.4-3.17 panel B). The CaR immunoreactivity observed (under reducing conditions) in wild-type CaR-transfected cells was consistent with that observed in this and other laboratories previously (McCormick et al 2010; Lazarus et al 2011) namely 140 and 160 kDa bands with higher molecular weight oligomers exhibited in some gels.

At least eight of the histidine mutants exhibited CaR immunoreactivity indistinguishable from wild-type CaR-reactivity, namely, CaR\(^{H134V} \), CaR\(^{H192V} \), CaR\(^{H359V} \), CaR\(^{H377V} \), CaR\(^{H429V} \), CaR\(^{H463V} \), CaR\(^{H495V} \) and CaR\(^{H766V} \) (Panel B, Figures 3.3, 3.4, 3.9, 3.10, 3.12, 3.13, 3.15 and 3.16). Mutants CaR\(^{H312V} \) and CaR\(^{H413V} \) exhibited reduced expression of both 140 and 160 kDa CaR proteins (Panel B, Figures 3.7 and 3.12), while mutants CaR\(^{H254V} \), CaR\(^{H344V} \) and CaR\(^{H463V/H466V} \) had slightly reduced 160
kDa bands and increased levels of 140 kDa (Panel B, Figures 3.5 3.8 and 3.13). In contrast, two CaR histidine mutants, namely CaR\textsubscript{H41V} and CaR\textsubscript{H595V}, expressed very poorly indeed with very little 160 kDa (mature) CaR exhibited and thus these are shown separately in Figures 3.18 and 3.19 and discussed in section 3.4.3.

3.4.2 Characterisation of the extracellular calcium sensitivity of the CaR histidine mutants expressed abundantly in HEK-293 cells.

In order to confirm the functional activity of each of the CaR mutants, HEK-293 cells were transiently transfected with one of the mutants and then loaded with Fura2 and examined by epifluorescence microscopy. The effect of increasing Ca\textsuperscript{2+} concentration on the HEK-293 cell Ca\textsuperscript{2+} concentration was then tested and where possible the Ca\textsuperscript{2+} concentration-dependency of each response was evaluated i.e. to establish the EC\textsubscript{50} for Ca\textsuperscript{2+} on each mutant CaR at pH 7.4. This was done to ensure that the receptors had neither lost nor gained any functional activity as a result of the mutation, since the subsequent pH experiments were substantially dependent on the Ca\textsuperscript{2+}-sensitivity being unaffected by each mutation.

Those fourteen CaR histidine mutants that expressed abundantly (i.e. not including CaR\textsubscript{H41V} and CaR\textsubscript{H595V}) were exposed to various concentrations of Ca\textsuperscript{2+} (0.5-10 mM) for 15 minutes and the changes in fluorescence ratio were quantified as area under the curve (AUC) per minute. All values were then normalised to the maximal response for the wild-type CaR in order that any deleterious effect of each mutation on either EC\textsubscript{50} or the maximal response could be detected. The only two exceptions to this strategy were mutants CaR\textsubscript{H41V} and CaR\textsubscript{H595V} which both exhibited low protein expression of the 160kDa CaR band, as well as little Ca\textsuperscript{2+} mobilisation in preliminary experiments (not shown) in response to modest Ca\textsuperscript{2+} concentrations and thus are discussed separately in section 3.4.3. For the remaining 14 CaR histidine mutants that did exhibit CaR immunoreactivity and responsiveness, the data are shown in Figures 3.3-3.16 with mutants arranged by location of the histidine residue in ascending order (i.e. from CaR\textsubscript{H134V} to CaR\textsubscript{H766V}).

For wild-type CaR, 1.5 mM Ca\textsuperscript{2+} exhibited little or no effect whereas there was a substantial response in 2.5 mM Ca\textsuperscript{2+}. With 3.5 mM Ca\textsuperscript{2+}, the CaR response was close to half-maximal, reaching the full maximum at 10 mM Ca\textsuperscript{2+}. The calculated EC\textsubscript{50} for all wild-type CaR responses was 4.3 ± 0.3 mM Ca\textsuperscript{2+} (N=16; Figure 3.17, Table 3.1). Next, of those CaR mutants that exhibited equivalent CaR expression to
wild-type, namely CaR$^{H134V}$ (Figure 3.3), CaR$^{H359V}$ (Figure 3.9), CaR$^{H377V}$ (Figure 3.10), CaR$^{H463V}$ (Figure 3.13) and CaR$^{H495V}$ (Figure 3.15), the Ca$^{2+}$, concentration-dependency was also comparable to wild-type CaR responses. That is, the sigmoidal curves were not shifted significantly to either left or right and the maximal responses were also unchanged.

Mutants CaR$^{H192V}$ (Figure 3.4), CaR$^{H338V}$ (Figure 3.7) and CaR$^{H429V}$ (Figure 3.12) did exhibit Ca$^{2+}$ concentration-effect curves that appeared right-shifted, however, the calculated EC$_{50}$ values were not significantly increased for CaR$^{H338V}$ (Figure 3.7) and CaR$^{H429V}$ (Figure 3.12); only the EC$_{50}$ for CaR$^{H192V}$ (Figure 3.4) was significantly increased relative to wild-type CaR (**P<0.01 by One-way ANOVA, Dunnett’s post-hoc test). In contrast, mutant CaR$^{H766V}$ (Figure 3.16) appeared to exhibit a left-shifted concentration-effect curve relative to wild-type CaR however, the resulting EC$_{50}$ was not significantly different. Mutant CaR$^{H312V}$ (Figure 3.6) was unchanged in comparison to WT however, CaR$^{H413V}$ (with reduced expression of both CaR bands) (Figure 3.11) appeared to exhibit left-ward shifted curves though again the EC$_{50}$ values were not significantly from wild-type CaR. In those mutants that exhibited reduced expression of the 160 kDa band; CaR$^{H463V/H466V}$ (Figure 3.14) was slightly right-ward shifted and CaR$^{H254V}$ (Figure 3.5) slightly left-ward shifted, though again neither EC$_{50}$ value was significantly different from wild-type. CaR$^{H344V}$ (Figure 3.8) was right was shifted and EC$_{50}$ was significantly altered (*P<0.05 by One-way ANOVA Dunnett’s post-hoc test P<0.05).

The important point to note from these data is that for the mutants described in this section, 3.5 mM Ca$^{2+}$ elicited robust Ca$^{2+}$ mobilisation that was largely oscillatory in nature at that Ca$^{2+}$ concentration. As such, 3.5 mM Ca$^{2+}$ was deemed a suitable concentration to employ in experiments testing the effect of the mutations on pH$_{o}$-sensitivity relative to wild-type CaR and the rationale for this is more fully described in the Discussion (section 3.5).
Table 3.1. EC$_{50}$ values and relative maximal responses for the CaR histidine mutants versus wild-type. The EC$_{50}$ values for CaR$^{H192V}$ and CaR$^{H344V}$ were significantly greater than for wild-type CaR (**P<0.001 and *P<0.05 respectively by One-way ANOVA, Dunnett’s post-hoc test). The remaining mutants had EC$_{50}$ values that were not significantly different from control and all of the receptor mutants exhibited maximal responsiveness that was not significantly different from wild-type CaR (Kruskal-Wallis, Dunn’s multiple comparison test).

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Figure 3.3. Validation of the CaR mutant His134Val. A) Forward primer used to generate the CaR$^{H134V}$ mutation, followed by i) in-house sequencing data confirming identity of the resulting mutant vector and ii) the resulting predicted protein sequence. B) Representative CaR and β-actin immunoblots obtained using lysates of HEK-293 cells transiently transfected with either wild-type (WT) CaR or CaR$^{H134V}$. C) Ca$^{2+}_{o}$ concentration-effect relationship for the same cells assayed for changes in Ca$^{2+}_{i}$ (Fura2 ratio). Data were normalised against mean maximal WT response. N=6.
Figure 3.4. Validation of the CaR mutant His192Val. CaR sequencing (A) and expression (B) data are shown for CaR_{H192V} in the same way as described for CaR_{H134V} in the legend to Figure 3.3 C) Ca^{2+}_{o} concentration-effect relationship for the same cells assayed for changes in Ca^{2+}_{i} (Fura2 ratio). Data were normalised against mean maximal WT response. N=7.
**Figure 3.5. Validation of the CaR mutant His254Val.** CaR sequencing (A) and expression (B) data are shown for CaR\textsuperscript{H254V} in the same way as described for CaR\textsuperscript{H134V} in the legend to Figure 3.3 C) \(\text{Ca}^{2+}\) concentration-effect relationship for the same cells assayed for changes in Ca\textsuperscript{2+} \text{ i} (Fura2 ratio). Data were normalised against mean maximal WT response. N=7.
Figure 3.6. Validation of the CaR mutant His312Val. CaR sequencing (A) and expression (B) data are shown for CaR$^{H312V}$ in the same way as described for CaR$^{H134V}$ in the legend to Figure 3.3 C) Ca$^{2+}$ concentration-effect relationship for the same cells assayed for changes in Ca$^{2+}$ (Fura2 ratio). Data were normalised against mean maximal WT response. N=5.
Figure 3.7. Validation of the CaR mutant His338Val. CaR sequencing (A) and expression (B) data are shown for CaR\textsuperscript{H338V} in the same way as described for CaR\textsuperscript{H134V} in the legend to Figure 3.3 C) Ca\textsuperscript{2+} concentration-effect relationship for the same cells assayed for changes in Ca\textsuperscript{2+ (Fura2 ratio)}. Data were normalised against mean maximal WT response. N=4.
**Figure 3.8. Validation of the CaR mutant His344Val.** CaR sequencing (A) and expression (B) data are shown for CaR$^{H344V}$ in the same way as described for CaR$^{H134V}$ in the legend to Figure 3.3 C) Ca$^{2+}$o concentration-effect relationship for the same cells assayed for changes in Ca$^{2+}$i (Fura2 ratio). Data were normalised against mean maximal WT response. N=7.

**A**

**H344V Primer:**
Forward: 5’ CGTCCAGGAAAGTCTGTCCACAATGGT 3’

**Confirmed Sequencing data:**
CGTCCAGGAAAGTCTGTCCACAATGGT

**Resulting Peptide sequence:**
- **wild-type:** LKKVHPRKSV**H**NGFAKEFWEE
- **mutant:** LKKVHPRKSVY**V**NGFAKEFWEE

**B**

![Image of CaR sequencing and expression data for CaR$^{H344V}$](image)

**C**

![Image of Ca$^{2+}$o concentration-effect relationship](image)
**Figure 3.9. Validation of the CaR mutant His359Val.** CaR sequencing (A) and expression (B) data are shown for CaR^{H359V} in the same way as described for CaR^{H134V} in the legend to Figure 3.3 C) Ca^{2+} concentration-effect relationship for the same cells assayed for changes in Ca^{2+}i (Fura2 ratio). Data were normalised against mean maximal WT response, N=8.
Figure 3.10. Validation of the CaR mutant His377Val. CaR sequencing (A) and expression (B) data are shown for CaR\textsuperscript{H377V} in the same way as described for CaR\textsuperscript{H134V} in the legend to Figure 3.3 C) \( [\text{Ca}^{2+}]_o \) concentration-effect relationship for the same cells assayed for changes in \( \text{Ca}^{2+} \) (Fura2 ratio). Data were normalised against mean maximal WT response. N=8.
**Figure 3.11. Validation of the CaR mutant His413Val.**

CaR sequencing (A) and expression (B) data are shown for CaR\textsuperscript{H413V} in the same way as described for CaR\textsuperscript{H134V} in the legend to Figure 3.3 C) Ca\textsuperscript{2+} concentration-effect relationship for the same cells assayed for changes in Ca\textsuperscript{2+} (Fura2 ratio). Data were normalised against mean maximal WT response, N=6.

**A**

**H413V Primer:**
```
Forward: 5' GACCCCTTACATAGATTACACGG\textbf{GT}TTACGGATATCCTACAATGTG 3'
```

**Confirmed Sequencing data:**
```
GACCCCTTACATAGATTACACGG\textbf{GT}TTACGGATATCCTACAATGTG
```

**Resulting Peptide sequence:**

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<th>mutant</th>
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<td>SVETPYIDYTHLRISYNVYLA</td>
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</table>

**B**

![Image of Western Blot](image)

**C**

![Image of Graph](image)
Figure 3.12. Validation of the CaR mutant His429Val. CaR sequencing (A) and expression (B) data are shown for CaR\textsuperscript{H429V} in the same way as described for CaR\textsuperscript{H134V} in the legend to Figure 3.3 C) \(\text{Ca}^{2+}_o\) concentration-effect relationship for the same cells assayed for changes in \(\text{Ca}^{2+}_i\) (Fura2 ratio). Data were normalised against mean maximal WT response. N=3.
Figure 3.13. Validation of the CaR mutant His463Val. CaR sequencing (A) and expression (B) data are shown for CaR<sub>H463V</sub> in the same way as described for CaR<sub>H134V</sub> in the legend to Figure 3.3 C) Ca<sup>2+</sup> concentration-effect relationship for the same cells assayed for changes in Ca<sup>2+</sup> (Fura2 ratio). Data were normalised against mean maximal WT response. N=6.
Figure 3.14. Validation of the CaR mutant His463Val/His466Val. CaR sequencing (A) and expression (B) data are shown for CaR<sub>H463V/H466V</sub> in the same way as described for CaR<sub>H134V</sub> in the legend to Figure 3.3 C) Ca<sup>2+</sup> concentration-effect relationship for the same cells assayed for changes in Ca<sup>2+</sup> (Fura2 ratio). Data were normalised against mean maximal WT response. N=9.
Figure 3.15. Validation of the CaR mutant His495Val. CaR sequencing (A) and expression (B) data are shown for CaR\textsuperscript{H495V} in the same way as described for CaR\textsuperscript{H134V} in the legend to Figure 3.3 C) Ca\textsuperscript{2+}\textsubscript{o} concentration-effect relationship for the same cells assayed for changes in Ca\textsuperscript{2+}\textsubscript{i} (Fura2 ratio). Data were normalised against mean maximal WT response. N=3.

A

\textbf{H495V Primer:}
Forward: 5’ ATCCATCATCAACTGG\textsuperscript{GT}CCTCTCCCAGAGGATG 3’

\textbf{Confirmed Sequencing data:}
ATCCATCATCAACTGG\textsuperscript{GT}CCTCTCCCAGAGGATG

\textbf{Resulting Peptide sequence:}
\begin{tabular}{ll}
wild-type & LVGNYSIINWHLSPEDGSIVF \\
mutant & LVGNYSIINWVLSPEDGSIVF \\
\end{tabular}

B

\textbf{C}

\textbf{CaR activity}

\begin{tabular}{ll}
% CaR & activity \\
\end{tabular}

\begin{tabular}{ll}
\textbf{H495V} & \\
\textbf{wild-type} & \\
\end{tabular}

\begin{tabular}{ll}
[Ca\textsuperscript{2+}\textsubscript{o}] (mM) & \\
0 & 2 \\
4 & 6 \\
8 & 10 \\
\end{tabular}

\textbf{WT H495V}

\begin{tabular}{ll}
kDa & \\
160 & 140 \\
45 & \\
\end{tabular}

\textbf{CaR}

\textbf{β-actin}
Figure 3.16. Validation of the CaR mutant His766Val. CaR sequencing (A) and expression (B) data are shown for CaR$_{H766V}$ in the same way as described for CaR$_{H134V}$ in the legend to Figure 3.3 C) Ca$^{2+}$o concentration-effect relationship for the same cells assayed for changes in Ca$^{2+}$i (Fura2 ratio). Data were normalised against mean maximal WT response. N=9.
3.4.3 Characterisation of the extracellular calcium sensitivity of the CaR histidine mutants CaR<sub>H41V</sub> and CaR<sub>H595V</sub>

As previously mentioned, the mutants CaR<sub>H41V</sub> and CaR<sub>H595V</sub> exhibited little or no 160kDa mature CaR immunoreactivity or responsiveness to moderate Ca<sup>2+</sup><sub>o</sub> concentrations. Indeed, CaR<sub>H41V</sub> failed to respond to any Ca<sup>2+</sup><sub>o</sub> concentrations <20 mM even in the presence of the calcimimetic NPS-R467, hereinafter called R467 (1 µM) although transient calcium mobilisation was observed in the presence of 20 mM Ca<sup>2+</sup><sub>o</sub> plus R467 (Figure 3.18). However, CaR<sub>H41V</sub> did then elicit Ca<sup>2+</sup><sub>i</sub> oscillations in 30 mM Ca<sup>2+</sup><sub>o</sub> supplemented with 1 µM R467.

CaR<sub>H595V</sub> did not respond to physiological concentrations of Ca<sup>2+</sup><sub>o</sub> either but did exhibit Ca<sup>2+</sup><sub>i</sub> oscillations in the presence of 5 mM Ca<sup>2+</sup><sub>o</sub> supplemented with 1 µM R-467 though still failed to reach its maximal response in 30 mM Ca<sup>2+</sup><sub>o</sub> (Figure 3.19). As a result, it was not possible to determine the EC<sub>50</sub> values for Ca<sup>2+</sup><sub>o</sub> for either CaR<sub>H41V</sub> or CaR<sub>H595V</sub> and thus subsequent experiments with these mutants were performed using 30 mM Ca<sup>2+</sup><sub>o</sub> + 1 µM R467, or, 5 mM Ca<sup>2+</sup><sub>o</sub> + 1 µM R467 respectively.

Figure 3.17. Concentration-effect curve for WT CaR. Representative trace of the Ca<sup>2+</sup><sub>o</sub> concentration-effect relationship for HEK-293 cells transiently transfected with WT human CaR, assayed for changes in Ca<sup>2+</sup><sub>i</sub> (Fura2 ratio) in single cells (orange and purple) and ‘global’ response (black). N=16.
Figure 3.18. Validation of the CaR mutant His41Val. A) Forward primer used to generate the CaR\textsuperscript{H41V} mutation, followed by i) in-house sequencing data confirming identity of the resulting mutant vector and ii) the resulting predicted protein sequence. B) Representative anti-CaR and β-actin immunoblots obtained using lysates of HEK-293 cells transiently transfected with either wild-type (WT) CaR or CaR\textsuperscript{H41V}. C) Ca\textsuperscript{2+} concentration-effect relationship for the same cells assayed for changes in Ca\textsuperscript{2+} (Fura2 ratio). Data were normalised against mean maximal WT response. N=9.
Figure 3.19. Validation of the CaR mutant His595Val. CaR sequencing (A) and expression (B) data are shown for CaR<sup>H595V</sup> in the same way as described for CaR<sup>H41V</sup> in the legend to Figure 3.18 C. Ca<sup>2+</sup> eff concentration-effect relationship for the same cells assayed for changes in Ca<sup>2+</sup> (Fura2 ratio). Data were normalised against mean maximal WT response. N=9.
3.4.4 Determination of the extracellular pH sensitivity of the CaR histidine mutants that respond to modest extracellular calcium concentrations.

Having identified 14 CaR histidine mutants capable of eliciting robust Ca\(^{2+}\)\(_i\) mobilisation in response to 3.5 mM Ca\(^{2+}\)\(_o\), the effect of altering pH\(_o\) was then examined in these mutant receptors. Specifically, the effect of 0.2 pH\(_o\) unit changes on CaR-induced Ca\(^{2+}\)\(_i\) mobilisation, at a single Ca\(^{2+}\)\(_o\) concentration (3.5 mM) was tested for each mutant in turn. For wild-type CaR and all of the CaR mutants except for CaRH41V and CaR\(^{H595V}\), increasing Ca\(^{2+}\)\(_o\) from 0.5 to 3.5 mM induced Ca\(^{2+}\)\(_i\) oscillations as before. Then decreasing the pH\(_o\) from 7.4 to 7.2 attenuated the responses; oscillations were still observed however they tended to have lower amplitudes and reduced frequencies (quantification not shown). Next, increasing the pH\(_o\) from 7.4 to 7.6 then potentiated the Ca\(^{2+}\)\(_i\) mobilisation induced by 3.5 mM Ca\(^{2+}\)\(_o\); with oscillations increased in frequency and amplitude, or even changed into sustained responses. The pH\(_o\)–dependent effects were then fully reversible, with a return to pH 7.4 causing a restoration of the original response. These data were then quantified and the change in the area-under-the-curve per min (∆AUC) for each CaR mutant compared to that of wild-type CaR both for acidosis and alkalosis conditions. This revealed that all of the CaR histidine mutants described here remained sensitive to pathophysiological changes in pH\(_o\). Representative traces for each of these 14 CaR mutants are shown in Figures 3.20-3.33 together with a bar chart showing the quantification of the relative effects of pathophysiological acidosis or alkalosis on CaR responsiveness.
Figure 3.20. The effect of $\text{pH}_o$ changes on $\text{Ca}^{2+}\text{o}$-induced $\text{Ca}^{2+}\text{i}$ mobilisation for mutation $\text{CaR}^{\text{H134V}}$. A) Representative trace showing $\text{CaR}^{\text{H134V}}$-transfected HEK-293 cells stimulated with 3.5 mM $\text{Ca}^{2+}\text{o}$ and then exposed to pathophysiological changes in $\text{pH}_o$. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=9. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.21. The effect of $pH_o$ changes on $Ca^{2+}_o$-induced $Ca^{2+}_i$ mobilisation for mutation $CaR^{H192V}$. A) Representative trace showing $CaR^{H192V}$-transfected HEK-293 cells stimulated with 3.5 mM $Ca^{2+}_o$ and then exposed to pathophysiological changes in $pH_o$. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N≥6. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.22. The effect of $pH_o$ changes on $Ca^{2+}_o$-induced $Ca^{2+}_i$ mobilisation for mutation $CaR^{H254V}$. A) Representative trace showing $CaR^{H254V}$-transfected HEK-293 cells stimulated with 3.5 mM $Ca^{2+}_o$ and then exposed to pathophysiological changes in $pH_o$. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N≥6. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.23. The effect of pH\textsubscript{o} changes on Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation for mutation CaR\textsuperscript{H312V}. A) Representative trace showing CaR\textsuperscript{H312V}\textsubscript{I} transfected HEK-293 cells stimulated with 3.5 mM Ca\textsuperscript{2+}\textsubscript{o} and then exposed to pathophysiological changes in pH\textsubscript{o}. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=6. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.24. The effect of pH\textsubscript{o} changes on Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation for mutation Ca\textsubscript{R}\textsubscript{H338V}. A) Representative trace showing Ca\textsubscript{R}\textsubscript{H338V}-transfected HEK-293 cells stimulated with 3.5 mM Ca\textsuperscript{2+}\textsubscript{o} and then exposed to pathophysiological changes in pH\textsubscript{o}. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=6. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.25. The effect of pH₀ changes on Ca²⁺₀-induced Ca²⁺ᵢ mobilisation for mutation CaR¹³₄⁴V. A) Representative trace showing CaR¹³₄⁴V-transfected HEK-293 cells stimulated with 3.5 mM Ca²⁺₀ and then exposed to pathophysiological changes in pH₀. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=6. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.26. The effect of pH₀ changes on Ca²⁺₀-induced Ca²⁺ᵢ mobilisation for mutation CaR⁵⁵⁹V. A) Representative trace showing CaR⁵⁵⁹V-transfected HEK-293 cells stimulated with 3.5 mM Ca²⁺₀ and then exposed to pathophysiological changes in pH₀. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=6. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.27. The effect of pH\textsubscript{o} changes on Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation for mutation CaR\textsuperscript{H377V}. A) Representative trace showing CaR\textsuperscript{H377V}-transfected HEK-293 cells stimulated with 3.5 mM Ca\textsuperscript{2+}\textsubscript{o} and then exposed to pathophysiological changes in pH\textsubscript{o}. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=7. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.28. The effect of pH$_o$ changes on Ca$^{2+o}$-induced Ca$^{2+}$i mobilisation for mutation CaR$^{H413V}$. A) Representative trace showing CaR$^{H413V}$ transfected HEK-293 cells stimulated with 3.5 mM Ca$^{2+o}$ and then exposed to pathophysiological changes in pH$_o$. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=9. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.29. The effect of $\text{pH}_o$ changes on $\text{Ca}^{2+}_o$-induced $\text{Ca}^{2+}_i$ mobilisation for mutation $\text{CaR}^{H429V}$. A) Representative trace showing $\text{CaR}^{H429V}$ transfected HEK-293 cells stimulated with 3.5 mM $\text{Ca}^{2+}_o$ and then exposed to pathophysiological changes in $\text{pH}_o$. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=7. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.30. The effect of $pH_o$ changes on $Ca^{2+}_o$-induced $Ca^{2+}_i$ mobilisation for mutation $CaR^{H463V}$. A) Representative trace showing $CaR^{H463V}$-transfected HEK-293 cells stimulated with 3.5 mM $Ca^{2+}_o$ and then exposed to pathophysiological changes in $pH_o$. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N≥6. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.31. The effect of $pH_o$ changes on $Ca^{2+}_o$-induced $Ca^{2+}_i$ mobilisation for mutation $CaR^{H463V/H466V}$. A) Representative trace showing $CaR^{H463V/H466V}$-transfected HEK-293 cells stimulated with 3.5 mM $Ca^{2+}_o$ and then exposed to pathophysiological changes in $pH_o$. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=7. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.32. The effect of pH\textsubscript{o} changes on Ca\textsuperscript{2+\textsubscript{o}}-induced Ca\textsuperscript{2+\textsubscript{i}} mobilisation for mutation CaR\textsuperscript{H495V}. A) Representative trace showing CaR\textsuperscript{H495V} transfected HEK-293 cells stimulated with 3.5 mM Ca\textsuperscript{2+\textsubscript{o}} and then exposed to pathophysiological changes in pH\textsubscript{o}. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N≥8. B) Quantification of the changes in AUC/min is shown as a bar chart.
Figure 3.33. The effect of $pH_o$ changes on $Ca^{2+}_o$-induced $Ca^{2+}_i$ mobilisation for mutation $CaR^{H766V}$. A) Representative trace showing $CaR^{H766V}$ transfected HEK-293 cells stimulated with 3.5 mM $Ca^{2+}_o$ and then exposed to pathophysiological changes in $pH_o$. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=9. B) Quantification of the changes in AUC/min is shown as a bar chart.
Together, Figures 3.20-3.33 indicate that none of these histidine residues contribute significantly to CaR pH₀ sensitivity. It was noted however that the pH₀ sensitivities of CaR\textsuperscript{H429V} and CaR\textsuperscript{H495V} appeared marginally decreased and so these two residues are considered further in Section 3.4.6. Nevertheless, as single mutations the pH₀ sensitivity of CaR\textsuperscript{H429V} and CaR\textsuperscript{H495V} was not significantly different to wild-type CaR.

3.4.5 Determination of the extracellular pH sensitivity of the CaR histidine mutants CaR\textsuperscript{H41V} and CaR\textsuperscript{H595V}.

Next, the pH₀ sensitivities of the two remaining CaR histidine (i.e. the loss-of-function) mutants were tested. For CaR\textsuperscript{H41V} and CaR\textsuperscript{H595V}, Ca\textsuperscript{2+} mobilisation was induced using 30 mM Ca\textsuperscript{2+}₀ plus 1 µM R-467 or 5 mM Ca\textsuperscript{2+}₀ plus 1 µM R-467 respectively followed by changes in pH₀ tested previously. However, as for the other mutants, decreasing pH₀ from 7.4 to 7.2 inhibited CaR responsiveness for CaR\textsuperscript{H41V} (Figure 3.34) and CaR\textsuperscript{H595V} (Figure 3.35) whereas increasing pH₀ from 7.4 to 7.6 potentiated receptor responsiveness. The treatments necessary to induce Ca\textsuperscript{2+} mobilisation were supramaximal with respect to wild-type CaR, and thus are unsuitable for direct comparison to the effects of 3.5 mM Ca\textsuperscript{2+}₀ (i.e. ~EC\textsubscript{50}). Therefore for these two mutants, the effects of changing pH₀ were compared to their prior responsiveness in pH 7.4. As a result, quantification of the changes in area under the curve in response to changes in pH₀ reveal that both CaR\textsuperscript{H41V} and CaR\textsuperscript{H595V} retain their pH₀ sensitivity (CaR\textsuperscript{H41V}, **P<0.01 and CaR\textsuperscript{H595V}, *P<0.05 by paired t-test vs pH₀ 7.4). Therefore, I found no evidence that any of the 16 extracellular histidine residues contribute to the pH₀ sensitivity of CaR.
Figure 3.34. The effect of pH\textsubscript{o} changes on Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation for mutation CaR\textsuperscript{H41V}. A) Representative trace showing CaR\textsuperscript{H41V} transfected HEK-293 cells stimulated with 3.5 mM Ca\textsuperscript{2+}\textsubscript{o} and then exposed to pathophysiological changes in pH\textsubscript{o}. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=9. B) Quantification of the changes in AUC/min is shown as a bar chart. **P<0.01 by paired t-test N=4.
Figure 3.35. The effect of pH\textsubscript{o} changes on Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation for mutation CaR\textsuperscript{H595V}. A) Representative trace showing CaR\textsuperscript{H595V} transfected HEK-293 cells stimulated with 3.5 mM Ca\textsuperscript{2+}\textsubscript{o} and then exposed to pathophysiological changes in pH\textsubscript{o}. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=9. B) Quantification of the changes in AUC/min is shown as a bar chart. *P<0.05 by paired t-test N=4.
3.4.6 Characterisation of the extracellular calcium and pH sensitivity of the CaR double histidine mutant, CaR$^{H429V/H495V}$.

Having failed to identify a sole histidine residue as being responsible for CaR pH$_o$ sensitivity, I next co-mutated the two histidine residues that appeared to have produced the largest trend reductions in pH$_o$ sensitivity, namely CaR$^{H429V}$ and CaR$^{H495V}$ (see Figure 3.36). The objective was to determine whether the combined trend reductions in pH$_o$ sensitivity could achieve statistical significance. Therefore, the vector containing the CaR$^{H429V}$ mutant was then mutated again using the primer introducing the CaR$^{H495V}$ mutation so as to produce CaR$^{H429V/H495V}$. Successful introduction of the H495V mutation in CaR$^{H429V}$ was confirmed by sequencing (Figure 3.36 panel A). Next, the double mutant was transfected into HEK-293 cells and its abundant expression confirmed by immunoblotting (Figure 3.36 panel B). Exposure of cells transfected with CaR$^{H429V/H495V}$ to increasing concentrations of Ca$^{2+}_o$ again elicited a Ca$^{2+}_o$ concentration-dependent increase in Ca$^{2+}_i$ mobilisation with a calculated EC$_{50}$ value similar to wild-type CaR (5.0 ± 0.4 mM N=7 vs. 4.3 ± 0.3, N=16, NS; Figure 3.36 panel C).
A H429V Primer:
  Forward: 5’ AGCAGTCTACTCCATTGCCGTGCGCTTGCAAGATATATAT 3’
H495V Primer:
  Forward: 5’ ATTCATCATCAACTTGGGTCTCTCCCCAGAGGATG 3

Confirmed Sequencing data:
ATTGCCC GTGCCCT....TCAACTGGG GTCTCTC

Resulting Peptide sequence:
  wild-type LAVYSIAHALQDI...YSIINWHLSPEEDG
  mutant LAVYSIAVALQDI...YSIINWLSPEDG

B

C

Figure 3.36. Validation of the CaR mutant His429Val/His495Val. A) Forward primer used to generate the CaR^{H429V/H495V} mutation, followed by i) in-house sequencing data confirming identity of the resulting mutant vector and ii) the resulting predicted protein sequence. B) Representative CaR and β-actin immunoblots obtained using lysates of HEK-293 cells transiently transfected with either wild-type (WT) CaR or CaR^{H429V/H495V}. C) Ca^{2+}_o concentration-effect relationship for the same cells assayed for changes in Ca^{2+}_i (Fura2 ratio). Data was normalised against mean maximal WT response. N=9.
Having validated the expression and responsiveness of CaR$^{H429V/H495V}$, its sensitivity to pH$_o$ was then tested as before, in response to 3.5 mM Ca$^{2+}_o$. However, the pH$_o$ responsiveness of CaR$^{H429V/H495V}$ was not significantly different to that of wild-type CaR (Figure 3.37). In fact, the mean responses to changes in pH$_o$ were marginally elevated suggesting that the marginal decreases seen for the individual mutations were not real and were merely due to experimental variability. With no other histidine mutants exhibiting any trend changes in pH$_o$ sensitivity, it was decided not to generate any further double histidine mutations.
Figure 3.37. The effect of pH\textsubscript{o} changes on Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation for mutation CaR\textsuperscript{H429V/H495V}. A) Representative trace showing CaR\textsuperscript{H429V/H495V} transfected HEK-293 cells stimulated with 3.5 mM Ca\textsuperscript{2+}\textsubscript{o} and then exposed to pathophysiological changes in pH\textsubscript{o}. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=7. B) Quantification of the changes in AUC/min is shown as a bar chart.
3.4.7 Characterisation of the extracellular calcium and pH sensitivity of the free cysteine mutant, CaR$^{C482S}$.

Finally, having established that extracellular histidine residues are unlikely to contribute pH$_o$ sensitivity to the CaR, it was noted that the pK values of free cysteine sidechains (8.5) are also similar to the physiological pH and thus could contribute to pH$_o$ sensitivity. All but one of the extracellular cysteine residues in CaR are normally oxidised and involved in intra- or intermolecular binding leaving only CaR$^{C482S}$ assumed to be free (Zajickova et al., 2007). As a result it was decided to mutate this residue and test its pH$_o$ sensitivity as before.

Sequencing of the resulting CaR$^{C482S}$ mutant confirmed successful introduction of the mutation (Figure 3.38 panel A) and its expression in HEK-293 cells was confirmed by immunoblotting as before (Figure 3.38 panel B). Then exposure of cells transfected with CaR$^{C482S}$ to increasing concentrations of Ca$^{2+}_o$ resulted in concentration-dependent Ca$^{2+}_i$ mobilisation with an EC$_{50}$ not significantly different to that for wild-type CaR (Figure 3.38 panel C).
A \textbf{C}_{482}S \text{Primer:} \\\n\text{Forward:} \ 5' \text{GGTGACCTTTGATGAGAGTG} \text{GTGACCTGTTGGG} \ 3' \\\n
\textbf{Confirmed Sequencing data:} \\\n\text{GGTGACCTTTGATGAGAGTG} \text{GTGACCTGTTGGG} \\\n
\textbf{Resulting Peptide sequence:} \\\n\text{wild-type} \quad \text{NMGEQVTDFDECGDLVGNYSII} \\\n\text{mutant} \quad \text{NMGEQVTDFE5GDLVGNYSII} \\\n
\textbf{Figure 3.38. Validation of the CaR mutant Cys482Ser.} A) Forward primer used to generate the CaR\textsuperscript{C482S} mutation, followed by i) in-house sequencing data confirming identity of the resulting mutant vector and ii) the resulting predicted protein sequence. B) Representative anti-CaR and \(\beta\)-actin immunoblots obtained using lysates of HEK-293 cells transiently transfected with either wild-type (WT) CaR or CaR\textsuperscript{C482S}. C) \(\text{Ca}^{2+}\text{o}\) concentration-effect relationship for the same cells assayed for changes in \(\text{Ca}^{2+}\) (Fura2 ratio). Data was normalised against mean maximal WT response. N=9.
Thus, having confirmed the responsiveness of CaRC482S to moderate Ca\textsuperscript{2+}\textsubscript{o} challenge, the effect(s) of pathophysiological changes in pH\textsubscript{o} were tested on the mutant as before. However, the pH\textsubscript{o} responsiveness of CaRC482S in the presence of 3.5 mM Ca\textsuperscript{2+}\textsubscript{o} was not significantly different to that of wild-type (Figure 3.39).

![Figure 3.39](image.png)

**Figure 3.39. The effect of pH\textsubscript{o} changes on Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation for mutation CaRC482S.** A) Representative trace showing CaRC482S transfected HEK-293 cells stimulated with 3.5 mM Ca\textsuperscript{2+}\textsubscript{o} and then exposed to pathophysiological changes in pH\textsubscript{o}. The trace shows two single cells (orange and purple) and the “global” response in the cell cluster (black). N=7. B) Quantification of the changes in AUC/min is shown as a bar chart.

3.4.8 Summary
Figure 3.40 below summarises the lack change in pH$_o$ responsiveness for any of the histidine or free cysteine residues examined.

Figure 3.40. Bar chart summarising the pH$_o$ sensitivity of all of the CaR histidine/cysteine mutants tested. Percentage stimulation in alkalosis (pH 7.6, upper panel) normalised against initial response to CaR stimulation in pH 7.4. Analysis by One-way ANOVA with Dunnett’s vs. WT $p>0.05$. The lower panel shows the equivalent responses to acidosis (pH 7.2), normalised against 1$^{st}$ 7.4 exposure. Analysis by One-way ANOVA vs. WT $p>0.05$. The dotted line indicates the pH$_o$-induced stimulation and inhibition for wild-type CaR and is shown to aid comparison.
3.5 Discussion

3.5.1 The effect of altered extracellular pH on the CaR in a heterologous expression system

It has been demonstrated previously that the potency of Ca$^{2+}$ for CaR is affected by the ambient pH$_o$. Quinn et al and Doroszewicz et al have both demonstrated that large i.e. 1 unit or greater changes in pH$_o$ significantly alter the CaR's sensitivity (Doroszewicz et al, 2005; Quinn et al, 2004). However, human blood pH is maintained between 7.35 and 7.45 and since pH is a logarithmic scale then a 1.0 unit change in fact represents a tenfold change in proton concentration and such a change in blood pH would certainly be lethal (Edwards, 2008). This led us to ask whether the CaR would respond to smaller, pathophysiologically relevant changes in pH$_o$. Metabolic acidosis and secondary hyperparathyroidism (SHPT) are common consequences of chronic kidney disease (CKD) and senescence and thus it is worth considering whether the current data may provide a mechanistic link between the two and thus a potential therapeutic intervention.

Experiments performed in this lab previously (Figures 1.3 and 1.4) showed that small, pathophysiologically relevant (0.2) pH$_o$ unit changes significantly alter CaR sensitivity; an increase in proton concentration causing the receptor to become less sensitive to cations, while alkalosis rendered CaR more sensitive. It was observed using the Ca$^{2+}$ indicator FURA2 that pH$_o$ alters CaR sensitivity in stably transfected CaR-HEK cells and bovine parathyroid cells (McCormick, 2008), an effect known to be mediated by activation of the G$_{q/11}$/PLC pathway. CaR-induced ERK phosphorylation and actin polymerisation were also pH$_o$-sensitive (McCormick, 2008), indicating that this sensitivity is not limited to G$_{q/11}$-mediated Ca$^{2+}$ mobilisation, but appears to be true for a number of G protein-coupled signals downstream of the CaR. Although these findings do not eliminate the possibility that changing pH$_o$ acts upon other cellular targets simultaneously, the simplest explanation for the data is that the alterations in pH$_o$ act via a common mechanism, namely altered agonist potency at the CaR. Furthermore, that the same pH$_o$ effects are also seen in bovine parathyroid chief cells in which CaR is expressed endogenously further supports the idea that CaR is the principal mediator of the proton-induced modulation. Indeed there is data from the laboratory of our collaborator suggesting that PTH secretion may also be affected by alterations in pH$_o$ in cultured human parathyroid cells (unpublished results).
Furthermore, Lopez et al observed in dogs that when ionised calcium was clamped, metabolic acidosis in which blood pH was decreased from 7.41 to 7.26 induced a significant increase in PTH levels (Lopez et al, 2004; Lopez et al, 2002). Likewise, when blood pH was increased by metabolic alkalosis, by 0.22 pH units, PTH was significantly decreased (Lopez et al, 2003). There were no changes in plasma sodium, magnesium or phosphate levels during alkalosis or acidosis (Lopez et al, 2002; Lopez et al, 2003). During EGTA-induced hypocalcaemia, PTH levels returned to normal, in both groups. These experiments show that despite ionised calcium levels remaining unchanged, changes of ≈ 0.2 pH unit in the blood significantly affect PTH secretion. These whole animal experiments demonstrated the need for us to examine small pathophysiological (0.2 pH unit) changes in pH, in vitro, so as to determine the mechanism explaining their observations.

Similar results have been reported in other studies using human and animal models. Bichara et al found significant correlation between intact PTH levels and blood proton or bicarbonate concentration (Bichara et al, 1990). Then, in healthy humans and recurrent nephrolithiasis patients, acute acid load caused a transient increase in PTH within two hours that was reversed after four hours (Houillier et al, 1996). Serum total and ionised calcium concentrations were unchanged in controls and kidney stone patients (Houillier et al, 1996). Also, as we age, our GFR decreases and we become mildly acidotic and exhibit moderately increased PTH secretion (Frassetto & Sebastian, 1996; Frassetto et al, 1996). The link between CKD and metabolic acidosis is well established [reviewed in (Frassetto & Hsu, 2009; Kovacic & Roguljic, 2003; Kraut & Madias, 2011)]; impaired proximal tubule function leads to reduced H+ secretion via the sodium proton exchanger NHE3 (Pereira et al, 2009).

Another issue to consider is whether by changing pHo, we are also altering pHi and thus somehow affecting CaR downstream signalling. However, further data from this lab (McCormick, 2008) has shown that using the pHi-sensitive dye BCECF, altering pHo does not alter pHi in CaR-HEK or bovine PT cells, over this time scale; therefore the effect of the altered pHo must be mediated extracellularly. Quinn et al showed that pre-incubating cells in solutions at either pH 6.5 or 8.5 and then switching to the other pH caused a change in pHi that took 15 minutes to reach steady-state (Quinn et al, 2004). However, Quinn et al then went on to show that there was no significant difference in the subsequent EC50 (for Ca2+i) between cells incubated in the test pH or pre-incubated in a buffer 2.0 pH units different (Quinn et al, 2004). This evidence (Quinn et al, 2004) together with the current data
suggests that pH\textsubscript{o} exerts its effect on the ECD of the CaR rather than by modifying intracellular signalling and could therefore be either influencing agonist binding, or causing a conformational change in the receptor.

The question remains though how the CaR responds to changes to pH\textsubscript{o} \textit{in vivo}. Under physiological conditions, the CaR is exposed to multiple agonists and agonist-buffering agents. In order to determine if buffering of Ca\textsuperscript{2+} and H\textsuperscript{+} could compensate for changes to pH\textsubscript{o}, we repeated the same experiments in the presence of 5% albumin.

### 3.5.2 Potential effect of altered calcium binding to albumin following changes in pH\textsubscript{o}

In the blood, calcium is found either free-ionised or bound to a buffering agent, for example citrate, phosphate, or bicarbonate, with the majority being bound to albumin (Baker & Worthley, 2002; Oberleithner \textit{et al}, 1982; Toffaletti \textit{et al}, 1977). Acidosis could cause calcium to become displaced by protons, thus increasing the free calcium concentration; Wang \textit{et al} found whole blood sample from healthy humans, a 0.015 mM change in ionised Ca\textsuperscript{2+} per pH unit per litre of albumin (Wang \textit{et al}, 2002). These minute changes in free ionised calcium could theoretically compensate for the acidosis by increasing the agonist concentration in the blood.

However, in the current study, the presence of 5% (w/v) albumin, i.e. representing the physiological albumin concentration, failed to overcome the apparent pH\textsubscript{o}-sensitivity of the CaR. Pathophysiologically relevant 0.2 pH unit (to 7.2 or 7.6) changes in pH\textsubscript{o} still caused a significant potentiation or inhibition of Ca\textsuperscript{2+} release respectively versus 7.4 at a single Ca\textsuperscript{2+} o. This demonstrates that over this pH range the effect of changing pH\textsubscript{o} on CaR activity is still greater than any effect of calcium buffering and displacement. Although these experiments do not perfectly mimic the \textit{in vivo} buffering of calcium in blood, it should still be noted that the concentration of albumin used (5%) is at the upper limit of albumin normally found in the blood (Kragh-Hansen & Vorum, 1993).

Kragh-Hansen & Vorum offer one explanation for this, they found that between pH 6.8 to 7.4 the calcium binding affinity of albumin was not altered significantly (Kragh-Hansen & Vorum, 1993). At pH 7.7 and greater calcium binding is increased, so that at pH 8 there is a four-fold increase in the association constant (Kragh-Hansen & Vorum, 1993). Interestingly, chronic metabolic acidosis is associated with
protein wasting and hypoalbuminaemia, the effect this has on ionised calcium concentration in the blood is unknown, however it might be expected that less calcium would be bound in this case (Ballmer et al, 1995).

3.5.3 Potential contribution of extracellular histidine residues to CaR pH sensitivity

Proton sensitivity has been demonstrated in a large number of proteins and the amino acid residue most commonly responsible for such pH sensitivity over the physiological range is histidine (Ghanouni et al, 2000; Sekler et al, 1996; Zong et al, 2001). As discussed previously, the histidine sidechain has the pK (6.4) that is closest to the physiological range (7.35-7.45) and therefore is likely to be more deprotonated than protonated at a physiological pH. There are a number of pH-sensitive membrane proteins whose proton-sensitivity has been ascribed to extracellular histidine residues and these are reviewed briefly here.

For example, the anion exchange protein 2 (AE2) regulates cell volume, pH and Cl- by exchanging Na+ -independently for Cl-/CO₃²⁻ (Stewart et al, 2007). Using chimeras, Sekler et al identified a histidine-rich motif in the cytoplasmic domain that confers pH sensitivity (Sekler et al, 1996). In 2007, Stewart et al demonstrated that using the histidine mutagen diethylpyrocarbonate (DEPC) it was possible to alter AE2 pH sensitivity (Stewart et al, 2007). The group identified two histidines in particular (His-I144 and His-I145) that were critical for exchanger pH sensitivity (Stewart et al, 2007). The idea that multiple histidine residues contribute to pH sensitivity could be shared by the CaR; as the CaR has multiple binding sites and exhibits cooperative agonist binding. Here the effect of mutating two residues, which showed reduced (but not significantly) sensitivity to pH₀ was assayed, but did not abolish pH₀ sensitivity.

Ovarian cancer G-protein coupled receptor 1 (OGR1) and, the closely related, GPR4 have both been identified at proton sensors. Originally described as a sphingosylphosphorylcholine receptor, Ludwig et al demonstrated (by IP formation) at pH ≥7.8 OGR1 was completely inhibited, the receptor was maximally active at pH 6.8; interestingly, at pH<6.8 the receptor becomes inhibited (Ludwig et al, 2003). Furthermore the group identified five histidine residues that confer pH₀ sensitivity (His-17, -20, -84, -269 -169). It was hypothesised that hydrogen bonds formed between the histidines stabilise the ECD inhibiting pH-dependent activation. In
acidic conditions, protonation destabilises the complex, causing the residues to repel, and allowing for an active confirmation. OGR1 is expressed in bone and nervous system; both OGR1 and GPR4 are expressed in the lung and kidney.

The purinergic receptor (P2X<sub>4</sub>) has a single histidine (His-286) which confers pH sensitivity (Clarke <em>et al</em>, 2000). Similarly to the CaR, reducing pH<sub>o</sub> inhibits current amplitude while alkalosis increased it. Sequential mutation of the four extracellular histidines to alanine demonstrated that three have function similar to WT but mutation of His-286 resulted in concentration-effect relationships that were resistant to changes in pH<sub>o</sub> (Clarke <em>et al</em>, 2000).

These and other examples of histidine(s) rendering a protein pH<sub>o</sub> sensitive indicated the need to investigate the sixteen extracellular histidines in human CaR as putative mediators of pH<sub>o</sub> sensitivity. The CaR has at least three postulated Ca<sup>2+</sup> binding sites, clusters of negatively charged amino acids in the VFT domain. Modelling the histidine residues by this lab in conjunction with Dr J Warwicker (University of Manchester) showed that there are a number of histidine residues exposed in the VFT. We originally hypothesised that these histidines are most likely to determine pH sensitivity due to their ability to become protonated over the physiological pH range (Figure 1.4 and Table 1). Increasing the acidity of the experimental buffer would protonate the histidines which could change the conformation of the calcium-binding sites.

Individual mutation of each of the 16 histidine residues in the ECD of human CaR demonstrated that none of the histidines individually contribute to pH sensitivity in the human CaR. Co-mutation of two histidines, CaR<sub>H429V</sub> and CaR<sub>H495V</sub>, whose responses were judged to be the most insensitive to pH<sub>o</sub> still did not abolish pH sensitivity. The experiments were conducted at a single Ca<sup>2+</sup> concentration (3.5 mM Ca<sup>2+</sup>); it was decided that to be truly pH<sub>o</sub>-insensitive a mutant must not change sensitivity in the presence of increasing or decreasing pH<sub>o</sub>. Using a single Ca<sup>2+</sup> concentration enabled us to compare receptors under the same conditions as they should all be activated to a similar degree. The two exceptions (CaR<sub>His41Val</sub> and CaR<sub>His595Val</sub>) showed no 160kDa immunoblot band and did not respond to physiological Ca<sup>2+</sup> and were dealt with separately in order to determine if they were pH<sub>o</sub> sensitive. A calcium concentration below the WT-CaR EC<sub>50</sub> value was used, as preliminary experiments indicated that this would better allow us to compare both alkaline and acidic responses during the same experiment. Using a higher Ca<sup>2+</sup> concentration made it difficult to perceive a change during alkalosis. The stimulation
by alkalosis is much smaller than the inhibition by acidosis; this may indicate that
the receptor is more susceptible to inhibition by protonation, than potentiation by
alkalosis. It may also reflect the fact that at pH 7.4 the histidine sidechain is already
more likely to be deprotonated than protonated and therefore larger increases in
pH may be required to evoke changes in Ca\textsuperscript{2+}; mobilisation equivalent in magnitude
to the decrease in mobilisation elicited by lowering pH\textsubscript{o}.

In discussions with Dr Geoffrey Hendy (McGill University, Canada), it was realised
that cysteine 482 is a free residue, which is unconserved in mammals and is not
involved in inter- or intramolecular binding, and therefore its –SH sidechain could
also be susceptible to deprotonation over the physiological pH range (Zajickova et
al, 2007). Furthermore, mutation of Cys-482 did not disrupt PI hydrolysis or
membrane expression (Fan et al, 1998; Ray et al, 1999). As a result, the pH
sensitivity of this site was also investigated however its mutation to serine also failed
to alter CaR pH\textsubscript{o}-sensitivity. For the remaining extracellular cysteine residues to
have even a theoretical role in pH\textsubscript{o} sensitivity they would need to become
spontaneously reduced under physiological conditions. However, there is no
evidence to date that such reductions might occur and in any case extracellular
conditions are generally much less reducing than intracellular conditions.

Thus together, the data presented in this thesis tend to suggest that neither the 16
extracellular histidine residues in CaR nor CaR\textsubscript{C482} are responsible for the receptor’s
pH\textsubscript{o} sensitivity. While it is possible that co-mutation of particular histidine residue
combinations may prove more successful, co-mutation of the two histidines that did
show the greatest promise failed to alter pH\textsubscript{o} sensitivity and therefore this does not
appear a promising strategy to pursue, especially given the potential numbers of
histidine combinations (including 240 possible paired combinations and 3,360
possible triple combinations).

3.5.4 Extracellular pH sensitivity exhibited by the mGluRs

With regards the homologous metabotropic glutamate receptor family, mGluR4a
has also been shown to be sensitive to changes in pH\textsubscript{o} (Levinthal et al, 2009).
Increasing acidity inhibits the receptor while alkalinity potentiates its agonist
response. Levinthal et al hypothesised that as the inhibition could not be
surmounted by increasing agonist concentration, the inhibition is noncompetitive,
and therefore the pH sensitivity was rendered either by a separate proton binding
site or nonconserved region (Levinthal et al, 2009). In contrast, no pH$_o$-sensitivity was found for mGluRs 1a, 5d or 8b. Nevertheless, with mGluR4a exhibiting pH$_o$-sensitivity I employed basic local alignment search tool (BLAST) and a constraint-based multiple protein alignment tool (COBALT) to identify conserved sequences between the human CaR and mGluRs (1a, 4a, 5d and 8b) (table 3.2) (Altschul et al, 1990; Papadopoulos & Agarwala, 2007). Interestingly, one histidine residue was conserved between all 5 receptors, namely histidine 41, whereas a second residue histidine 429 was conserved only between human CaR and mGluRs 1a and 4a. However, none were shared exclusively between the pH-sensitive receptors, CaR and mGluR4a. This suggests that histidine conservation cannot explain pH sensitivity between these class C GPCRs.

<table>
<thead>
<tr>
<th>CaR</th>
<th>mGluR1</th>
<th>mGluR4</th>
<th>mGluR5</th>
<th>mGluR8</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>PHFG</td>
<td>SVHQ</td>
<td>PVH---</td>
<td>SVHQ</td>
</tr>
<tr>
<td>429</td>
<td>IAHAL</td>
<td>MAHGL</td>
<td>MGHAL</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Conservation of histidine between CaR and mGluRs. The NIH Constraint-based multiple protein alignment tool (COBALT) was used to identify conserved histidine residues between Homo sapiens isoforms of CaR, mGluR1, 4, 5 and 8, with a 2 bit conservation setting. Histidine-41 was conserved between all the receptors and histidine-429 between only CaR, mGluR1 and 4.

3.5.5 pH sensitivity of non-elemental CaR agonists.

Although the potency of elemental CaR agonists is not subject to pH$_o$-mediated changes in their charge, the potencies of certain other CaR agonists may be influenced by pH$_o$. For example, Quinn et al used the polycation spermine to stimulate the CaR, and pH$_o$ sensitivity was reversed; receptor sensitivity was propagated by acidosis and inhibited by alkalosis (Quinn et al, 2004). The same phenomenon was demonstrated using aminoglycoside antibiotics (AGAs) which appeared to reverse pH sensitivity (McLarnon et al, 2002). Specifically, McLarnon et al showed that the CaR potency of tobramycin, which has 5 amine groups, was leftward shifted by increasing acidity, (McLarnon et al, 2002). The ability of positive ionic charges to overcome the inhibition by acidity implies that either these agonists have a separate binding site which is not affected by pH or the positive effect on the agonists charge is greater than the negative effect on the receptor itself. It would be interesting to know the effect of pH on L-amino acids, as they have been show to use an alternative binding site and are able to undergo protonation. For example, L-phenylalanine has only one free amine group that could be protonated, therefore it is
questionable whether the ionic strength could overcome the inhibition by pH. As the L-amino acids bind to a different site pH may not affect activity.

3.5.6 pH\textsubscript{0} sensitivity of CaR mutants

In order to determine the site responsible for pH sensitivity, Quinn et al investigated the activities of five CaR mutants expressing activating mutations (Quinn et al, 2004). It was stated that the activating mutations that had the greatest decrease in EC\textsubscript{50}, were least affected by pH\textsubscript{0} whereas those mutations with EC\textsubscript{50}s closest to that for WT-CaR were most affected by pH\textsubscript{0}. Quinn et al surmised that the increased activity of each mutant was due to increased pH sensitivity i.e. they would be more activated by alkalinity than would the WT-CaR (Quinn et al, 2004) however no information about expression was presented with this study.

Interestingly, Quinn et al demonstrates that the mutation CaR\textsuperscript{C129F} appears to be least affected by pH\textsubscript{0}, over the full experimental range (5.5-9.5) (Quinn et al, 2004). This site in the VFT is vital for dimerisation, and its mutation has previously been shown to cause a left-ward shift in the Ca\textsuperscript{2+} concentration-effect relationship (Ray et al, 1999; Zhang et al, 2001). Therefore, dimerisation and the close interaction of two ECDs might participate in pH\textsubscript{0} sensitivity.

Quinn and co-workers also showed that mutating the same residue to different amino acids can drastically alter the outcome (Quinn et al, 2004). Mutation of glutamate 127 to lysine, which is positive at physiological pH, causes the greatest change in EC\textsubscript{50}, and is the most activating, however mutation E127 to alanine, which is nontitratable, is only moderately activating (Quinn et al, 2004). The act of mutating glutamate, a negatively charged amino acid, with anything but aspartate is likely to alter CaR activity. Valine and serine are both nontitratable and should not be influenced by changes in pH\textsubscript{0}, therefore they are good candidates for site-directed mutagenesis of putative pH\textsubscript{0} sensitive residues. Nevertheless it should be noted that valine is ‘smaller’ than histidine and could change receptor conformation, or interactions with neighbouring amino acids.

3.5.7 Physiological relevance of extracellular pH sensing

As previously discussed, the discovery of specialised proton-sensors (OGR1 and GRP4), had led to increased interest in pH homeostasis. A number of body systems
such as bone, the immune system and cardiovascular system are sensitive to fluctuations in blood pH and thus it is clear that proton homeostasis is vital to our health. The current study is part of a wider study proving for the first time that the \( \text{pH}_o \) sensitivity of the CaR provides for \( \text{pH}_o \)-sensitivity of the parathyroid gland itself.

The CaR is expressed in pancreatic exocrine acini and luminal ductal cells and endocrine islet of Langerhans (Bruce et al, 1999). It was suggested that in the luminal ducts, the CaR senses calcium released with pancreatic enzymes, which stimulates bicarbonate and ductal fluid secretion (Bruce et al, 1999; Racz et al, 2002). Activation of the CaR by \( \text{Ca}^{2+} \) stimulates \( \text{HCO}_3^- \) and fluid secretion neutralising and diluting the \( \text{Ca}^{2+} \). This would protect against pancreatic stone formation and pancreatitis (Bruce et al, 1999; Racz et al, 2002). Furthermore, sufferers of FHH (inactivating CaR mutation), have a predisposition to pancreatic stone formation (Bruce et al, 1999). This would generate positive feedback and neutralise the acidic more efficiently and providing an anti-lithogenic benefit in the pancreas (Hegyi et al, 2011).

One site of CaR expression in the kidney is in the CCD, where it induces luminal proton secretion via the \( \text{H}^+ \)-ATPase (Farajov et al, 2008). In the IMCD principal cells, the CaR limits vasopressin-induced water reabsorption via AQP2 (Sands et al, 1997). In this context, pH sensitivity would appear to be counterproductive as secreting excess protons would decrease CaR sensitivity and therefore proton secretion, increasing the occurrence of kidney stones (Renkema et al, 2009). However, increasing proton concentration in the urine would also increase the free calcium concentration by dissolving calcium salts which could compensate for the acidosis and activate the CaR maintaining the nephrolithiasis protective mechanism.

Chronic metabolic acidosis is associated with chronic kidney disease, ageing and even a Western diet (Frassetto et al, 1996; Kopple et al, 2005; Kovacic & Roguljic, 2003; Lemann, 1999). In CKD and during senescence, reduced kidney function leads to gradual acidosis (see Introduction). There are also a number of acute causes of acidosis including; diarrhoea, diabetes mellitus and diuretics (Edwards, 2008). Renal-dependent chronic metabolic acidosis is caused by decreased proton excretion and bicarbonate reabsorption. Bicarbonate is primarily reabsorbed in the PCT via an electroneutral process; carbonic acid is formed within the cells which dissociates to \( \text{H}^+ \) and \( \text{HCO}_3^- \). Protons leave the cells across the apical membrane via NH3, a sodium/proton exchanger; the bicarbonate and sodium then leave the cell across the basolateral membrane via the NBC2 (Pereira et al, 2009; Rodríguez Soriano, 2002).
Bicarbonate reabsorption is under the control of a number of factors including calcium and PTH concentration (Rodríguez Soriano, 2002). The inability to excrete protons in exchange for bicarbonate causes acidification.

Meta-analysis of twenty-six studies found a positive correlation between age and blood proton concentration; and age dependent decrease glomerular filtration rate (GFR) (Frassetto & Sebastian, 1996; Frassetto et al, 1996). A Western diet may also facilitate acidosis, high vegetable to animal protein ratio diets are known to support acidosis (Frassetto & Kohlstadt, 2011; Kurtz et al, 1983). Vegetables are high in bicarbonates which reduces the net acid load, protecting against chronic acidosis. Interestingly countries with a high vegetable to animal protein diet had the lowest hip fracture incidence (Frassetto et al, 2000). Over time, low blood pH can cause bone decalcification, as the body compensates for the increased protons by releasing calcium salts.

The biggest reservoir of calcium in the body is bone. CKD is strongly associated with osteodystrophy, which causes bone demineralisation, osteopenia and metastatic calcification (Kovacic & Roguljic, 2003). Patients with CKD cannot catalyse the synthesis of 1,25 dihydroxyvitamin D3, which leads to hypocalcaemia and SHPT, in combination with metabolic acidosis these factors cause calcium to be reabsorbed from the bone (Cibulka & Racek, 2007; Kovacic & Roguljic, 2003). Vascular calcification in CKD patients affects the medial wall and tunica media artery walls (Goodman et al, 2004). This restricts the artery’s ability to stretch and respond to changes in blood pressure (Goodman et al, 2004). The process of bone demineralisation is mediated by osteoblasts and osteoclasts; culturing mouse osteoclasts and osteoblasts demonstrated that in acidic conditions, osteoblasts are inhibited while osteoclasts are activated (Krieger et al, 1992). According to the current hypothesis, the metabolic acidosis of CKD may further act to inhibit the parathyroid CaR and promote PTH secretion which would exacerbate SHPT and drive further bone demineralisation and vascular calcification. Interestingly, Oka et al have recently reported that pre-haemodialysis bicarbonate blood concentrations are significantly associated with coronary artery calcification in haemodialysis patients (Oka et al, 2012). Therefore, it will be interesting to know whether treating acidosis more aggressively in haemodialysis than is the case at present might improve cardiovascular outcomes in CKD.

Like acidosis, metabolic alkalosis is also a serious pathology. Ingestion of large quantities of calcium and alkali can develop milk-alkali syndrome (Felsenfeld &
Levine, 2006). Alkalosis has been shown to activate the CaR and suppress PTH secretion in dogs (Doroszewicz et al, 2005; Lopez et al, 2003; Quinn et al, 2004). In combination with hypercalcaemia, alkalosis would further suppress PTH secretion leading to hypoparathyroidism.

3.5.8 Summary

In conclusion, CaR activity is sensitive to pathophysiologically relevant (0.2 pH unit) changes in pH, which can affect PTH secretion. Albumin does not compensate for the increase in protons by increasing the free calcium concentration and none of the extracellular histidines or free cysteines are responsible for conferring pH sensitivity on CaR. Together, these observations may have relevance to a number of high incidence conditions that lead to chronic metabolic acidosis, including diabetes, hypertension and senescence. In combination with other factors, acidosis may increase PTH secretion and bone demineralisation potentially leading to vascular calcification and other complications. Knowledge of the progression of the disease may lead to improved therapeutics and improved prognosis.
Chapter 4

Calcium-sensing receptor intracellular signalling integration
4.1 Introduction

Although the involvement of PKC in CaR signalling has been well established (Bai et al., 1998b; Davies et al., 2007; Jiang et al., 2002; McCormick et al.; Racke & Nemeth, 1993), the function of the putative PKA sites and role of cAMP signalling in CaR function is less clear. In this chapter, the effects of cAMP and PKA modulators on Ca^{2+} mobilisation will be described and discussed. This will include determining the effects of mutating the putative PKA phosphorylation sites within CaR as well as altering G_{i}-mediated signalling.

4.2 Materials and Methods

CaR-HEK cells were cultured as detailed in section 2.1. Intracellular calcium imaging (section 2.6), immunoblotting (section 2.5) and site-directed mutagenesis (section 2.7) were performed as described in the sections named. Site-directed mutagenesis employed the primers listed in section 2.7.1 and the resulting mutations were sequenced to ensure identity. If appropriate unconstrained sigmoidal dose-response curve fitting was used.

4.3 Effect of increasing intracellular cAMP on CaR signalling in a heterologous expression system

4.3.1 Effect of forskolin addition during CaR-induced Ca^{2+} mobilisation

Gerbino et al published data which showed that in CaR-HEK cells stimulating cAMP generation, using PGE2, enhanced spermine-stimulated Ca^{2+} oscillations (Gerbino et al., 2005). To confirm and expand upon their findings, CaR-HEK cells loaded with the ratiometric dye, Fura2, were first incubated in buffer containing 0.5 mM Ca^{2+} and then exposed to buffer containing 3.5 mM Ca^{2+} which resulted in the induction of Ca^{2+} oscillations. Cells were then treated with forskolin (FSK) (10 µM) to activate adenylate cyclase (AC) and increase intracellular cAMP. Acute treatment with FSK caused a significant increase in Ca^{2+} mobilisation; observed as an increase in amplitude and frequency of the oscillations (Figure 4.1 panel A), that continued until Ca^{2+} and FSK were removed. Quantification of the change in fluorescence ratio revealed a significant increase in CaR activity upon addition of FSK (* P<0.05 vs. 3.5 mM Ca^{2+} by paired t-test; N=5; Figure 4.1 panel B).
4.3.2 Effect of forskolin on CaR-induced Ca^{2+}i mobilisation at sub-threshold Ca^{2+}o concentrations

In 3.5 mM Ca^{2+}o, the G_{q}/PLC pathway is activated initiating Ca^{2+}i oscillations. However, at calcium concentrations that do not initiate Ca^{2+}i oscillations (<2 mM), it is known that G_{12/13} is active and Rho kinase is able to polymerise actin (Davies et al, 2006). This would imply that there are Ca^{2+}o concentrations capable of activating CaR signalling but not specifically Ca^{2+}i mobilisation, and thus that the CaR is somehow prevented, or inhibited, from initiating Ca^{2+}i oscillations. Therefore, it was
next determined whether increasing intracellular cAMP concentrations could permit the initiation of Ca\textsuperscript{2+1} oscillations even at low (<2 mM) Ca\textsuperscript{2+0} concentrations.

Here, I will refer to ‘threshold’ Ca\textsuperscript{2+0} concentrations as those which are the minimum necessary to elicit G\textsubscript{q/11}-mediated, CaR-induced Ca\textsuperscript{2+1} mobilisation, although it should be noted throughout that strictly speaking the true CaR threshold is lower given that CaR-induced actin polymerisation occurs at lower Ca\textsuperscript{2+0} concentrations. Therefore my use of the term will relate to Ca\textsuperscript{2+1} mobilisation unless otherwise stated. Then having defined the Ca\textsuperscript{2+0} threshold concentration, I will also refer to the Ca\textsuperscript{2+0} subthreshold concentration as being the highest Ca\textsuperscript{2+0} concentration in which ≈95% of cells remain unresponsive, that is, that fail to mobilise Ca\textsuperscript{2+1}.

Because of experimental variability between cells and buffers etc., it was necessary to determine empirically the Ca\textsuperscript{2+0} threshold concentration for each coverslip. To achieve this, CaR-HEK cells were exposed to decreasing Ca\textsuperscript{2+0} concentrations in 0.2 mM steps starting at 2 mM Ca\textsuperscript{2+0}. In 2 mM Ca\textsuperscript{2+0} it was normal to see ≈60% of cells exhibiting either transient or even oscillatory Ca\textsuperscript{2+1} mobilisation. Reducing Ca\textsuperscript{2+0} concentration to 1.8 or even to 1.6 mM decreased Ca\textsuperscript{2+1} mobilisation until ≈95% of cells were unresponsive. Even then, baseline 350/380 ratios were modestly elevated relative to 0.5 mM however this is seen even in non-transfected HEK-293 cells and thus is unlikely to be specifically CaR-dependent (Nemeth, 2004). Having established the threshold Ca\textsuperscript{2+0} concentration, cells were then returned to buffer containing 0.5 mM Ca\textsuperscript{2+0} concentration in order to re-equilibrate the cells for the experiment. After 3-5 minutes, the cells were then exposed to the empirical “subthreshold” Ca\textsuperscript{2+0} concentration (usually either 1.6 or 1.8 mM) for at least 3 minutes during which time it was confirmed that ≈95% of cells remain unresponsive. At this point, addition of FSK (10 µM) to the buffer resulted in significant Ca\textsuperscript{2+1} mobilisation; (**P<0.001 vs. threshold Ca\textsuperscript{2+0} by paired t-test, N=6; Figure 4.2). This suggests that forskolin somehow increases CaR-G\textsubscript{q/11} sensitivity thus permitting Ca\textsuperscript{2+1} mobilisation at subthreshold Ca\textsuperscript{2+0} concentrations.
**Figure 4.2. Forskolin-induced increase in Ca\textsuperscript{2+} oscillations in threshold Ca\textsuperscript{2+}.** A) Representative trace showing the Ca\textsuperscript{2+} changes (Fura2 ratio) in two single cells (orange and purple) and global cluster (black) of CaR-HEK cells. Cells exposed to a single Ca\textsuperscript{2+} concentration (threshold 1.6-1.8 mM) followed by forskolin (10 µM) exposure, B) Quantification of the change in area under the curve/min following the addition of FSK. ***P<0.001 by paired t-test, N = 6.

### 4.3.3 Effect of 1,9-dideoxyforskolin on CaR-induced Ca\textsuperscript{2+} mobilisation at subthreshold Ca\textsuperscript{2+} concentrations

In order to determine the specificity of the forskolin action on Ca\textsuperscript{2+} mobilisation, the experiment was repeated first using the inactive forskolin analogue, 1,9-dideoxyforskolin. In the presence of subthreshold Ca\textsuperscript{2+} concentration, exposure to 10 µM 1,9-dideoxyforskolin was without effect whereas subsequent exposure to 10 µM forskolin significantly increased Ca\textsuperscript{2+} mobilisation as before (**P<0.01 by repeated measures ANOVA, Dunnett’s post test, N=8; Figure 4.3).
Figure 4.3. Calcium oscillations are not potentiated using the forskolin inactive analogue 1,9-dideoxyforskolin. A) Representative trace of FURA-2 loaded CaR-HEK cells, exposed to subthreshold Ca\(^{2+}\)o and then 1,9 dideoxyforskolin (10 µM) was added. This was then washed out and cells were returned to subthreshold Ca\(^{2+}\)o only before being exposed to forskolin (10 µM). B) Quantification of the changes in AUC following each treatment. Comparing the treatments showed a significant increase in AUC with forskolin (\(** P<0.01\), Repeated measures ANOVA and Dunnett’s Post Hoc test. N=8).
4.3.4 Effect of forskolin on CaR-induced Ca^{2+}_i mobilisation at low Ca^{2+}_o concentration

To determine whether forskolin stimulates Ca^{2+}_i mobilisation regardless of Ca^{2+}_o concentration, CaR-HEK cells were next incubated in 0.5 mM Ca^{2+}_o concentration and then cotreated with 10 µM forskolin. At this low Ca^{2+}_o concentration, forskolin was largely without effect (Figure 4.4). The transient rise in Ca^{2+}_i concentration seen in Figure 4.4 most likely represents a change artefact as this was not observed in the replicates.

Figure 4.4. Forskolin-induced increase in Ca^{2+}_i oscillations in baseline Ca^{2+}_o. A) Representative trace showing the Ca^{2+}_i changes (Fura2 ratio) in two single cells (orange and purple) and global cluster (black) of CaR-HEK cells. Cells exposed to a single Ca^{2+}_o concentration (0.5 mM) followed by forskolin (10 µM).

4.3.5 Effect of forskolin on CaR-induced Ca^{2+}_i mobilisation as a function of concentration

To determine the concentration dependence of forskolin on CaR-induced Ca^{2+}_i mobilisation, CaR-HEK cells were next incubated in 3 mM Ca^{2+}_o concentration and then exposed to increasing concentrations of forskolin (10 nM-50 µM). Forskolin elicited a significantly greater increase in CaR-induced Ca^{2+}_i mobilisation when used at 10µM as opposed to 1µM (*** P<0.001, N=4; Figure 4.5). Increasing the forskolin concentration further to 50µM, was without additional effect (NS), confirming that 10µM forskolin is the optimal drug concentration.
**Figure 4.5. Effect of increasing forskolin concentrations on Ca\(^{2+}\) mobilisation in moderate Ca\(^{2+}\).** A) Example trace of CaR-HEK cells exposed to moderate Ca\(^{2+}\) (3 mM) followed by increasing forskolin concentration (10 nM-50 µM). Single cells (orange and purple) and ‘global’ response (black) lines. B) Data was normalised against zero forskolin results for each experiment. * P<0.05 by One-way ANOVA vs. 10 nM forskolin; N=4. R\(^2\) = 0.76.

4.3.6 **Effect of forskolin on CaR-induced Ca\(^{2+}\) mobilisation as a function of extracellular calcium concentration**

Next, to determine whether forskolin causes a general increase in CaR sensitivity to Ca\(^{2+}\) concentration, concentration-effect curves were generated for Ca\(^{2+}\) on Ca\(^{2+}\) mobilisation in the presence or absence of 10µM forskolin. In the presence of forskolin, there was a significant increase in Ca\(^{2+}\) potency for CaR-induced Ca\(^{2+}\) mobilisation (EC\(_{50}\), 3.0 ± 0.1 mM control, 2.3 mM ± 0.1 mM +forskolin; Figure 4.6)
as exhibited by a leftward shift in the concentration-effect curve. However, forskolin did not alter the maximal response of the Ca\textsuperscript{2+}, (at 5 mM) for Ca\textsuperscript{2+} mobilisation.

**Figure 4.6. Effect of forskolin on concentration–effect curve.** Panel A) example traces of i) Control CaR-HEK cells or ii) Forskolin (10 µM) treated CaR-HEK cells exposed to increasing concentrations of Ca\textsuperscript{2+} (0.5-5 mM), orange and purple single cells and 'global' response to changes in Ca\textsuperscript{2+}. Panel B) Response to Ca\textsuperscript{2+} changes normalised against wild-type maximal response (wild-type – black, forskolin treated – orange). EC\textsubscript{50} was significantly leftward shifted 3.0 ± 0.1 mM control vs. 2.3 ± 0.1 mM P<0.05, N=6.
4.3.7 Effect of 3-isobutyl-1-methylxanthine on CaR-induced Ca$^{2+}$ mobilisation at subthreshold Ca$^{2+}_o$ concentrations

The simplest explanation for the actions of forskolin described in Sections 4.3.2 and 4.3.3 is that it increases intracellular cAMP levels and that this somehow lowers the CaR Ca$^{2+}_o$ threshold for mobilising Ca$^{2+}_i$ mobilisation. Another way of increasing intracellular cAMP concentration is to suppress its breakdown by inhibiting phosphodiesterases (PDEs), using xanthenes for example. Therefore, I next tested the effect of 3-isobutyl-1-methylxanthine (IBMX) on CaR-induced Ca$^{2+}_i$ mobilisation at subthreshold Ca$^{2+}_o$ concentrations. As before, CaR-HEK cells were exposed to their subthreshold Ca$^{2+}_o$ concentration and then cotreated with the PDE inhibitor, IBMX (100 µM). The IBMX significantly increased CaR-induced Ca$^{2+}_i$ mobilisation at this subthreshold Ca$^{2+}_o$ concentrations (**P<0.001 vs. threshold Ca$^{2+}_o$ by paired t-test, N=8, Figure 4.7). Removal of IBMX resulted in the rapid return of Ca$^{2+}_i$ concentration to baseline levels again. That IBMX broadly mimics the effect of FSK on CaR threshold responsiveness further supports the idea that cAMP somehow sensitises the CaR to Ca$^{2+}_o$. 


Figure 4.7. IBMX-induced increase in calcium mobilisation in threshold Ca\textsuperscript{2+}. A) Example trace of Fura-2 loaded CaR-HEK cells exposed to 0.5 mM Ca\textsuperscript{2+} to establish baseline, this was increased to subthreshold Ca\textsuperscript{2+}, Ca\textsuperscript{2+} increased but no oscillations are observed. The PDE inhibitor IBMX (100 µM) was then added inducing Ca\textsuperscript{2+} oscillations. B) Quantification of the change in AUC comparing Ca\textsuperscript{2+} only and addition of IBMX. IBMX significantly increased AUC. *** P<0.001 by paired t-test, N= 8.

4.3.8 Effect of forskolin cotreatment on CaR-induced ERK phosphorylation

Phosphorylation of the MAP kinase ERK by MEK is a well established readout of CaR activation and is downstream not only of G\textsubscript{q}, but also G\textsubscript{i} (Kifor et al, 2001). As demonstrated above (section 4.3.2), increasing cAMP significantly increases Ca\textsuperscript{2+} mobilisation at Ca\textsuperscript{2+} concentrations ranging from sub-threshold up to sub-maximal (~1.6--3 mM). It has been found by this laboratory (unpublished observation) and shown by Davey et al that the Ca\textsuperscript{2+} concentration-effect relation for ERK phosphorylation is significantly right-ward shifted in comparison to that for Ca\textsuperscript{2+} mobilisation (Davey et al, 2012). Therefore, in order to determine if the effect of cAMP is specific for Ca\textsuperscript{2+} mobilisation or also observed for other CaR effectors, the
effect of FSK on Ca\textsuperscript{2+o}–induced ERK phosphorylation was then studied. Firstly, to determine at which concentration of Ca\textsuperscript{2+o} to test the FSK, CaR-HEK cells were exposed to a range of Ca\textsuperscript{2+o} concentrations from 0.5-5 mM and lysed with RIPA buffer and assayed for ERK phosphorylation using a phospho-specific anti-ERK antibody (pERK). The resulting pERK immunoblots showed little or no p42/44 kDa pERK immunoreactivity following exposure to 0.5, 1, 2 or 3 mM Ca\textsuperscript{2+o}, whereas phospho-protein bands were visible in lane with Ca\textsuperscript{2+o} ≥4 mM (Figure 4.8). Therefore, it was decided that FSK would be tested in the presence of 3 mM Ca\textsuperscript{2+o} to determine if FSK could elicit a response not caused by 3 mM alone.

Accordingly, CaR-HEK cells were exposed to 3 or 5 mM Ca\textsuperscript{2+o} in the presence or absence of 10\micro M FSK. As before, no pERK bands were detected in 3 mM Ca\textsuperscript{2+o} in the absence of forskolin, whereas forskolin cotreatment did elicit ERK phosphorylation (*P<0.05, N=5; Figure 4.8). In contrast, the response to 5 mM Ca\textsuperscript{2+o} was not elevated by forskolin cotreatment. Total ERK abundance was not elevated following 10-min exposure to either forskolin or increased Ca\textsuperscript{2+o} concentration (not shown). Together these data support the idea that increasing cAMP increases CaR sensitivity more generally and is not specific to Ca\textsuperscript{2+o} mobilisation.
4.3.9 Effect of chronic phorbol ester pretreatment on \( \text{Ca}^{2+}_{\text{o}} \)-induced \( \text{Ca}^{2+}_{\text{i}} \) mobilisation

It is well established that overnight exposure of most cells to the phorbol ester PMA elicits downregulation of the classic and novel PKC isotypes thus permitting acute experiments to be undertaken in the presence or absence of these proteins. Specifically, it has been previously shown by this laboratory that such chronic exposure of CaR-HEK cells to PMA increases CaR sensitivity to \( \text{Ca}^{2+}_{\text{o}} \) for the mobilisation of \( \text{Ca}^{2+}_{\text{i}} \) (Davies et al, 2007; Handlogten et al, 2001). As a result, the threshold concentration of \( \text{Ca}^{2+}_{\text{o}} \) for \( \text{Ca}^{2+}_{\text{i}} \) mobilisation is lowered following PMA pretreatment, presumably because there is less inhibitory phosphorylation by PKC on the CaR ICD including Thr-888 (McCormick et al, 2010).
Thus, to determine whether the forskolin response occurs via PKC signalling, CaR-HEK cells were chronically exposed to PMA (1 µM, 18-hours), and then treated with or without forskolin 10 µM at a threshold Ca\textsuperscript{2+}\textsubscript{o} concentration. Firstly, as expected PMA pretreatment lowered the threshold Ca\textsuperscript{2+}\textsubscript{o} concentration necessary for Ca\textsuperscript{2+}\textsubscript{i} mobilisation, to 1.4 mM in the experiment shown (Figure 4.9). It was noted that the Ca\textsuperscript{2+}\textsubscript{i} responses of the PMA-pretreated cells to increased Ca\textsuperscript{2+}\textsubscript{o} concentration were largely sustained in nature as opposed to the transient or oscillatory responses generally observed without PMA pretreatment. Next, for PMA-pretreated cells incubated in a threshold Ca\textsuperscript{2+}\textsubscript{o} concentration, addition of forskolin resulted in a further elevation in Ca\textsuperscript{2+}\textsubscript{i} mobilisation (**P<0.01 vs. non-PMA FSK treated cells, N=10; Figure 4.9). This effect was fully reversed upon removal of forskolin and elevated Ca\textsuperscript{2+}\textsubscript{o} concentration. Therefore, PMA pretreatment failed to abolish the stimulatory effect of forskolin.
4.3.10 Effect of pertussis toxin pretreatment on Ca\(^{2+}\)\(_{o}\)-induced Ca\(^{2+}\)\(_i\) mobilisation

CaR activation is known to result in \(G_i\)-mediated inhibition of AC with a resulting reduction on cytosolic cAMP levels. Therefore, in experiments examining the effect of forskolin in CaR-HEK cells at various \(Ca^{2+}_o\) concentrations, the cAMP levels will most likely be increased by the forskolin but inhibited by the \(G_i\) activation resulting in a competitive interaction between the two components. To examine the likely contribution to \(Ca^{2+}_i\) mobilisation of cAMP suppression by CaR-induced \(G_i\) activity, CaR-HEK cells were pretreated overnight with pertussis toxin (PTx) as this is known to block \(G_i\) signalling via ADP-ribosylation. Next, cells were exposed to increasing...
concentrations of $Ca^{2+}_o$ and the resulting changes in $Ca^{2+}_i$ mobilisation quantified (Figure 4.10) and compared to those from control cells not pre-exposed to PTx.

The resulting concentration-effect curves were normalised against the average maximal response of control CaR-HEK cells to high $Ca^{2+}_o$ concentration. It was found that for PTX-treated cells, the $E_{\text{max}}$ for $Ca^{2+}_0$–induced $Ca^{2+}_i$ mobilisation was significantly greater than for control cells (**$P<0.01$, 100% ± 14% cont. vs. 216% ± 21% PTx treated; $N=8$, Figure 4.10). In fact, the $EC_{50}$ for $Ca^{2+}_o$ was not significantly different in PTX-treated cells as compared to control cells ($EC_{50}$ 3.0 mM ± 0.3 control vs. 1.9 mM ± 0.1 PTx treated) but because of the significantly elevated $E_{\text{max}}$, the response to 1.6 mM was significantly greater in PTX-treated cells (*$P<0.05$ by one-way ANOVA. $N\geq4$) than in control cells.
Figure 4.10. Effect of pertussis-toxin on $\text{Ca}^{2+}$ concentration–effect curve. Panel A) example traces of i) Control CaR-HEK cells or ii) Pertussis-toxin (100 ng/ml) treated CaR-HEK cells exposed to increasing concentrations of $\text{Ca}^{2+}$ (0.5-5 mM), orange and purple single cells and ‘global’ response to changes in $\text{Ca}^{2+}$. Panel B) Response to $\text{Ca}^{2+}$ changes normalised against wild-type maximal response (wild-type – black, pertussis toxin treated – orange). * P<0.05 $E_{\text{MAX}}$ by One way ANOVA N=8.

Then, to determine whether forskolin responses can be increased further by removing G-mediated AC suppression, cells incubated overnight in the absence or presence of PTx were exposed to a threshold $\text{Ca}^{2+}$ concentration and then additionally to 10 µM forskolin. Interestingly however, PTx pretreatment did not further increase the forskolin response as might have been expected (Figure 4.11). However, whether a lower i.e. submaximal forskolin concentration would have achieved an enhanced effect following PTx was not further tested.
4.3.11 Effect of isoproterenol on CaR-induced Ca\textsuperscript{2+} mobilisation at subthreshold Ca\textsuperscript{2+}\textsubscript{o} concentrations

Having shown that the drug forskolin can increase CaR sensitivity at subthreshold Ca\textsuperscript{2+}\textsubscript{o} concentrations, it was then necessary to determine whether endogenous stimulation of cAMP generation elicits the same effect. For this, the β-adrenoceptor agonist isoproterenol was used in order to induce G\textsubscript{o}-mediated AC activation and cAMP generation but with causing Ca\textsuperscript{2+} mobilisation.
Fura2-loaded CaR-HEK cells were exposed to subthreshold Ca\textsuperscript{2+}\textsubscript{o} concentrations, as previously described. Addition of isoproterenol (100 nM) then significantly increased Ca\textsuperscript{2+}\textsubscript{i} mobilisation (**P<0.01 vs. threshold Ca\textsuperscript{2+}\textsubscript{o}, N=5; Figure 4.12) increase in Ca\textsuperscript{2+}\textsubscript{i} as measured by FURA2. To establish that this effect is due to the activation of the CaR and not to a direct effect of the isoproterenol, the calcilytic NPS-2143 (1 µM) was further added to the buffer and this significantly inhibited the CaR response (*P<0.05 vs. Iso. treatment.). Removal of isoproterenol returned the Ca\textsuperscript{2+}\textsubscript{i} concentration baseline levels again.

**Figure 4.12. Effect of isoproterenol and NPS-2143 on Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation.** A) Changes in calcium mobilisation were measured as previously described (FURA2) in CaR-HEK cells were exposed to isoproterenol (100 nM) followed by NPS2143 (1 µM), in threshold Ca\textsuperscript{2+}\textsubscript{o}. (* P<0.05 vs. Ca\textsuperscript{2+}\textsubscript{o} alone. # P<0.05 isoproterenol vs. NPS-2143 treatment by One-way ANOVA Tukey’s post-hoc test) N=6.
Having shown in 4.3.10 that PTx can potentiate Ca\textsuperscript{2+\_o}-induced Ca\textsuperscript{2+\_i} mobilisation, the isoproterenol experiment was repeated in cells pre-exposed overnight to PTx (100ng/ml) to attenuate CaR-induced G\textsubscript{i} activation. Subsequent addition of isoproterenol in threshold Ca\textsuperscript{2+\_o} concentrations significantly increased Ca\textsuperscript{2+\_i} concentration (Figure 4.13) (**P<0.01 vs PTx treated control, N=6) as before. Then subsequent cotreatment with NPS-2143 (1 \textmu M) again significantly decreased Ca\textsuperscript{2+\_i} (**P<0.01 vs. Iso. treated. N=6) completely abolishing the apparent response to isoproterenol. Therefore, cAMP generation via activation of endogenous G\textsubscript{s}-linked receptors also potentiates CaR responsiveness. Further analysis showed that the isoproterenol responses were not significantly different between PTx treated (Figure 4.12) and untreated (Figure 4.13) cells in the presence of threshold Ca\textsuperscript{2+\_o} concentrations (p>0.05. N\geq5).
Figure 4.13. Effect of isoproterenol and NPS-2143 on Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-mobilisation in CaR-HEK cells pretreated with pertussis toxin. A) CaR-HEK cells were pretreated with pertussis toxin (100 ng/ml) for 16 hours. Changes in calcium mobilisation was measured as previously described (FURA2) in CaR-HEK cells exposed to isoproterenol (100 nM) followed by NPS2143 (1 µM), in threshold Ca\textsuperscript{2+}. B) Quantification of changes in calcium mobilisation as area under the curve/min (*P<0.05 vs. Ca\textsuperscript{2+} alone. # P<0.05 isoproterenol vs. NPS2143 treatment by One-way ANOVA Tukey’s post-hoc test; N=6).
4.4 Investigation of the role of protein kinase A in CaR signalling

4.4.1 Detection of protein kinase A activity using a motif-specific anti-PKA phospho-substrate antibody

Having demonstrated a clear effect of cAMP on CaR-induced Ca\textsuperscript{2+} mobilisation, I next attempted to identify the subcellular target of the cAMP. The two main intracellular effectors for cAMP are PKA and EPAC as described in the Introduction. Indeed, the CaR has two putative PKA phosphorylation sites in its ICD which could therefore mediate a stimulatory effect of PKA directly on the receptor. Therefore, to determine whether PKA is being activated by forskolin and/or elevated Ca\textsuperscript{2+} concentration in CaR-HEK cells, I employed a motif-specific anti-PKA phospho-substrate monoclonal antibody that detects proteins phosphorylated on the PKA sequence (R-R-X-pS/pT). For this, CaR-HEK cells were equilibrated in 0.5 mM Ca\textsuperscript{2+}–containing buffer for 20 minutes. The cells were then exposed to buffers containing 1.8, 3 or 5 mM Ca\textsuperscript{2+} in the presence or absence of FSK, for a further 5 minutes (Figure 4.14). Cells were then lysed with modified RIPA buffer and immunoblotted with the anti-PKA phospho-substrate antibody as described in Materials and Methods. Firstly, following 10-min incubation in buffer containing 1.8 mM Ca\textsuperscript{2+}, the antibody detected a number of proteins on the 8% gel, most notably a 30 kDa protein as well as a range of proteins between 75 and 100 kDa (Figure 4.14). Cotreatment with forskolin then elicited an increase in the total phosphorylation of these PKA substrates (*P<0.05, N=5). This indicates therefore that 10\mu M forskolin treatment in these cells results in acute PKA activation. Forskolin also increased PKA substrate phosphorylation (+156 ± 51%) in each of three experiments in which cells were cotreated with 3 mM Ca\textsuperscript{2+} instead (* P<0.05; N=3).
Figure 4.14. Immunoblot using a phospho-PKA consensus site antibody in a range of Ca\text{\textsuperscript{2+}} concentrations and forskolin treatment in CaR-HEK cells. A, Representative immunoblot showing increased PKA phosphorylation in threshold and 3 mM Ca\text{\textsuperscript{2+}} upon addition of FSK. N\geq3. B i) Bar graph comparing forskolin-treated and untreated in 1.8 mM Ca\text{\textsuperscript{2+}}. *P<0.05 by Paired t-test, N=5. B ii) Bar chart comparing baseline (0.5 mM), moderate Ca\text{\textsuperscript{2+}} (3 mM) untreated and forskolin (10 µM) treated and high Ca\text{\textsuperscript{2+}} (5 mM). *P<0.05 by One-way ANOVA, N=3.
4.4.2 Effect of protein kinase A inhibition and forskolin cotreatment on CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation at moderate Ca\textsuperscript{2+}\textsubscript{o} concentration

Having established that forskolin potentiates CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation and elicits PKA-mediated substrate phosphorylation, I then used PKA-selective inhibitors to examine whether the positive functional effect of forskolin is PKA-dependent. For this, cells were exposed to either the H-89 (10\(\mu\)M) or KT-5720 (10\(\mu\)M) which represents the most commonly used PKA inhibitors. With cells still exposed to the PKA inhibitors, the cells were then incubated in 2.8 mM Ca\textsuperscript{2+}\textsubscript{o} and then finally cotreated with FSK (10 \(\mu\)M). However, the responses to forskolin were not significantly suppressed in response to either KT5720 (10 \(\mu\)M) (Figure 4.15) or H89 (10 \(\mu\)M) (Figure 4.16). Interestingly, although not specifically quantified there is some indication that KT5720 may have potentiated increased the Ca\textsuperscript{2+}\textsubscript{i} mobilisation elicited by raising Ca\textsuperscript{2+}\textsubscript{o} concentration however no such trend observation was made using H89 and thus this effect was not investigated further. Nevertheless, the experiment suggests that despite 10\(\mu\)M forskolin activating PKA in these cells (Section 4.4.1), the resulting PKA activity may not account for the positive functional effect of forskolin on Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation.
Figure 4.15. Effect of the PKA inhibitor KT5720 on moderate Ca\textsuperscript{2+\textsubscript{o}}-induced Ca\textsuperscript{2+\textsubscript{i}} mobilisation. A) Representative trace showing CaR-HEK cells were exposed to 3 mM Ca\textsuperscript{2+\textsubscript{o}} to having either been untreated or pretreated with (A) KT5720 for 20 minutes, FSK (10 µM) was then added. Two single cells (orange and purple) and global cluster of cells (black) are depicted. B) Quantification of the change in calcium mobilisation in area under the curve/min. p=0.28 by Student’s t-test N=6.
Figure 4.16. Effect of the PKA inhibitor H89 on moderate Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation. A) Representative trace showing CaR-HEK cells were exposed to 3 mM Ca\textsuperscript{2+}\textsubscript{o} to having either been untreated or pretreated with (A) H89 for 20 minutes, FSK (10 µM) was then added. Two single cells (orange and purple) and global cluster of cells (black) are depicted. B) Quantification of the change in calcium mobilisation in area under the curve/min. P=0.94 by Student’s t-test N=6.
4.4.3 Effect of protein kinase A inhibition and forskolin cotreatment on CaR-induced Ca^{2+}_i mobilisation at threshold Ca^{2+}_o concentration

Although PKA inhibition failed to significantly inhibit the forskolin-induced potentiation of Ca^{2+}_o-induced Ca^{2+}_i mobilisation, it was considered necessary to also test the PKA inhibitor effects at subthreshold Ca^{2+}_o concentration as well. Again however, the forskolin-induced decrease in Ca^{2+}_o threshold for Ca^{2+}_i mobilisation was unaffected by cotreatment with either KT5720 (Figure 4.17) or H89 (Figure 4.18)(P>0.05 by One-way ANOVA, N=6).

Figure 4.17. Effect of the PKA inhibitor KT5720 on subthreshold Ca^{2+}_o-induced Ca^{2+}_i mobilisation. A) Representative trace showing CaR-HEK cells were exposed to 3 mM Ca^{2+}_o to having either been untreated or pretreated with (A) KT5720 for 20 minutes, FSK (10 µM) was then added. Two single cells (orange and purple) and global cluster of cells (black) are depicted. B) Quantification of the change in calcium mobilisation in area under the curve/min. P>0.05 by Student’s t-test N=6.
4.4.4 Effect of PKA- and EPAC-selective cAMP analogues on Ca\textsuperscript{2+}_i mobilisation at moderate Ca\textsuperscript{2+}_o concentration

Thus far, intracellular cAMP levels have been increased indirectly using agents such as FSK, IBMX or isoproterenol. Gerbino et al reported using a cell-permeable cAMP analogue (Sp-8-Br-cAMP, 200 µM) to enhance Ca\textsuperscript{2+}_i signalling in CaR-HEK cells (Gerbino et al, 2005) and therefore, next I employed cell-permeable cAMP-analogues reported to specifically activate either PKA or EPAC in order to determine
whether the cAMP’s effects on Ca\textsuperscript{2+}\textsubscript{i} mobilisation occur via either pathway. These included the cell-permeable cAMP analogues, 6-Bnz-cAMP, reported to selectively activate PKA (Roscioni \textit{et al}, 2011), and, 8-pCPT-2\textsuperscript{\prime}-O-Me-cAMP (pCPT-cAMP), reported to be a selective activator for EPAC (Roscioni \textit{et al}, 2011). In the first series of experiments using these cAMP analogues, CaR-HEK cells were stimulated with 3 mM Ca\textsuperscript{2+}\textsubscript{o} buffer and then cotreated with either 100 \muM 6-Bnz-cAMP, or 100 \muM pCPT-cAMP for at least 20mins. The choice of analogue concentration was based on their successful use in other laboratories reported previously (Dzhura \textit{et al}, 2010; Harmati \textit{et al}, 2011; Huang \textit{et al}, 2008; Kang \textit{et al}, 2005; Lo \textit{et al}, 2011; Roscioni \textit{et al}, 2011). However, in the current study no significant change in Ca\textsuperscript{2+}\textsubscript{o}–induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation was observed in response to either the PKA- or EPAC-selective cAMP analogues (Figure 4.19). To test whether the lack of analogue responsiveness was due to inadequate cellular uptake the next experiments employed a 1-hour pre-exposure of the cells (to 100 \muM 6-Bnz-cAMP or pCPT-cAMP) to permit longer for the compounds to accumulate within the cells. Since this experimental design no longer permitted before-and-after comparisons, a second series of cells not exposed to the analogues were also used as controls. However, as before there was no significant difference in Ca\textsuperscript{2+}\textsubscript{o}–induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation between analogue-treated and untreated cells (Figure 4.20), despite the 1-hour pretreatment.
Figure 4.19. Acute treatment of CaR-HEK cells in the presence of moderate Ca\textsuperscript{2+} (3 mM). A i and ii) Changes in Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} mobilisation was measured (FURA2) in CaR-HEK cells were exposed to moderate Ca\textsuperscript{2+} (3 mM) following treatment with (i) 6BnzICcAMP (100 µM) or (ii) cptcAMP (100 µm) for 20 minutes, in 2 single cells (orange and purple) and ‘global’ response. B) Quantification of the changes in FURA2 ratio normalised against control response.
Figure 4.20. Chronic treatment of CaR-HEK cells in the presence of moderate Ca\textsuperscript{2+} (3 mM). A) Representative traces of CaR-HEK cells control (black line) or chronically exposed to 6Bnz-cAMP (100 µm) (orange line) or (ii) cptcAMP (100 µm)(purple line) for one hour. B) Quantification of the changes in area under the curve. NS by one-way ANOVA, N≥3.

Finally, despite failing to observe any effect of the PKA- or EPAC-selective analogues on Ca\textsuperscript{2+}–induced Ca\textsuperscript{2+}i mobilisation their effect on threshold Ca\textsuperscript{2+} was also tested. The rationale was that more subtle effects of the analogues may be observed at threshold Ca\textsuperscript{2+} concentration than at 3 mM Ca\textsuperscript{2+}. For this, CaR-HEKs were exposed to 1.8 mM Ca\textsuperscript{2+} and then exposed to either 100 µM 6-Bnz-cAMP, or, 100 µM pCPT-cAMP. However, again, neither cAMP-analogue elicited Ca\textsuperscript{2+}–induced Ca\textsuperscript{2+}i mobilisation at this threshold Ca\textsuperscript{2+}–concentration (not shown).

4.4.5 Characterisation of the CaR Ser-899 and Ser-900 mutants

Garrett et al predicted the presence of 2 PKA phosphorylation sites in the human CaR, at Ser-899 and Ser-900 (Garrett et al, 1995); the function of which have not been established. Having found no evidence that either pharmacological activation
or inhibition of PKA modulates Ca\textsuperscript{2+}\textsubscript{o}–induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation in CaR-HEK cells, it was next decided to test whether mutating the two PKA consensus sites in the CaR ICD, serine-899 or serine-900, would alter CaR signalling. For this, the two putative PKA sites were mutated to either alanine (non-phosphorylatable) or to the phosphomimetic amino acid aspartate (as described in Materials and Methods).

Figure 4.21 panel A shows the successful introduction of the mutations into CaR resulting in CaR\textsuperscript{S899A} and CaR\textsuperscript{S900A}. The mutant proteins when expressed transiently in HEK-293 cells exhibited CaR immunoreactivity consistent with wild-type CaR. Then the Ca\textsuperscript{2+}\textsubscript{o} concentration (0.5-10 mM) effect relationship for Ca\textsuperscript{2+}\textsubscript{i} mobilisation was tested for each mutant relative to wild-type CaR. However, there was no apparent difference in CaR sensitivity observed between CaR\textsuperscript{S899A}, CaR\textsuperscript{S900A} and wild-type control. Specifically, there was no significant difference in the calculated EC\textsubscript{50} values or maximal responses of these mutant receptors relative to wild-type CaR (Table 4.1).

Next, figure 4.22 shows the equivalent experiments as above but for the phosphomimetic mutations CaR\textsuperscript{S899D} and CaR\textsuperscript{S900D}. Specifically, amino acid identity was confirmed by sequencing (Panel A) and protein expression equivalent to that of wild-type CaR was shown for (Panel B). When exposed to increasing Ca\textsuperscript{2+}\textsubscript{o} concentration the increase in Ca\textsuperscript{2+}\textsubscript{i} mobilisation was equivalent in CaR\textsuperscript{S900D} relative to wild-type receptor, whereas CaR\textsuperscript{S899D} exhibited greater Ca\textsuperscript{2+}\textsubscript{o} potency than in wild-type CaR, with the significantly decreased EC\textsubscript{50} value (2.6 ± 0.1 mM vs 3.0 ± 0.1 mM control, P<0.05) shown in Table 4.1. In contrast, the maximal responses were unchanged between mutants and wild-type CaR (P>0.05 E\textsubscript{max} WT 100 ± 10%. 93 ± 9%).

Finally, two separate dual PKA site CaR mutants were generated, the first where both residues were mutated to alanine (CaR\textsuperscript{S899A/S900A}) and the other in which the two sites were mutated to aspartate (CaR\textsuperscript{S899D/S900D}). Again, DNA sequencing (Figure 4.23, panel A) and immunoblotting (panel B) confirmed the successful mutation and expression of the two mutants relative to wild-type CaR. When the action of these two double mutations on Ca\textsuperscript{2+}\textsubscript{i} mobilisation were then tested, intriguingly they both exhibited heightened receptor responsiveness (panel C) as revealed by their significantly decreased EC\textsubscript{50} values for Ca\textsuperscript{2+}\textsubscript{o} (Table 4.1).

Specifically, CaR\textsuperscript{S899D/S900D} exhibited a significantly decreased EC\textsubscript{50}, although no more than for CaR\textsuperscript{S899D} alone (2.6 mM ± 0.1; P<0.01). Then CaR\textsuperscript{S899A/S900A} also
exhibited decreased EC\textsubscript{50} for Ca\textsuperscript{2+} \textsubscript{o} (2.6 mM ± 0.2; P<0.05) despite neither CaR\textsubscript{S899A} nor CaR\textsubscript{S900A} exhibiting increased sensitivity (Table 4.1).

**Figure 4.21 Validation and Ca\textsuperscript{2+} \textsubscript{o} sensitivity of the CaR mutant CaR\textsubscript{S899A} and CaR\textsubscript{S900A}.** A) Forward Primer used to generate the mutation, sequencing of the mutation retrieved from in-house service and the generated mutation in the CaR protein sequence. B) Representative immunoblot staining against total-CaR and β-actin. C) Transiently transfected HEK-293 cells were exposed to increasing Ca\textsuperscript{2+} \textsubscript{o} (0.5-10 mM), changes in Ca\textsuperscript{2+} \textsubscript{i} were measured (Fura2 ratio). Data was normalised against mean maximal WT response. N=6.
Figure 4.22. Validation and Ca\textsuperscript{2+} sensitivity of the CaR mutant CaR\textsuperscript{S899D} and CaR\textsuperscript{S900D}. A) Forward Primer used to generate the mutation, sequencing of the mutation retrieved from in-house service and the generated mutation in the CaR protein sequence. B) Representative immunoblot staining against i) Total CaR and ii) β-actin. C) Transiently transfected HEK-293 cells were exposed to increasing Ca\textsuperscript{2+} (0.5-10 mM), changes in Ca\textsuperscript{2+} were measured (Fura2 ratio). Data was normalised against mean maximal WT response. N=6.

\textbf{S899D Primer:}  
Forward - 5’-TCTCCGCAAGCGGG\textbf{GA}CACGAGCCTTGAG-3’

\textbf{Confirmed Sequencing data:}  
TCTCCGCAAGCGGG\textbf{GA}CACGAGCCTTGAG

\textbf{Resulting Peptide sequence:}  

\begin{center}
\begin{tabular}{ll}
wild-type & LRRSNVSRKRSSSLGGSTGSTR \\
mutant & LRRSNVSRKR\textbf{D}SSSLGGSTGSTR \\
\end{tabular}
\end{center}

\textbf{S900D Primer:}  
Forward - 5’-CCGCAAGCGGTCC\textbf{GA}ACGCTTGAGGC-3’

\textbf{Confirmed Sequencing data:}  
CCGCAAGCGGTCC\textbf{GA}ACGCTTGAGGC

\textbf{Resulting Peptide sequence:}  

\begin{center}
\begin{tabular}{ll}
wild-type & LRRSNVSRKRSSSLGGSTGSTR \\
mutant & LRRSNVSRKR\textbf{D}SSSLGGSTGSTR \\
\end{tabular}
\end{center}
**A**  
**SS899/900AA Primer:**  
Forward - 5’-CTCCGCAAGCGGGCCGCGCCAGCTTGGAGG-3’

**Confirmed Sequencing data:**  
CTCCGCAAGCGGGCCGCGCCAGCTTGGAGGCTC

**Resulting Peptide sequence:**  
- Wild-type: LRRSNVSRKKSSSLGGSTGSTR  
- Mutant: LRRSNVSRKKASSSLGGSTGSTR

**SS899/900DD Primer:**  
Forward - 5’-CGTCTCCGCAAGCGGGACGGACGGACGGCCAGCTTGGAGG-3’

**Confirmed Sequencing data:**  
CGTCTCCGCAAGCGGGACGGACGGACGGCCAGCTC

**Resulting Peptide sequence:**  
- Wild-type: LRRSNVSRKKSSSLGGSTGSTR  
- Mutant: LRRSNVSRKKDDSSLGGSTGSTR

**B**  
[Immunoblot image showing WT, SS/AA, and SS/DD bands at kDa 160, 140, and 45]

**C**  
[Graph showing % CaR activity vs. [Ca^{2+}]_o (mM) with SS/DD and wild-type curves, data normalized against mean maximal WT response, N=6]

**Figure 4.23. Validation and Ca^{2+}_o sensitivity of the CaR mutant CaR^{S899A/S900A} and CaR^{S899D/S900D}**.  
A) Forward Primer used to generate the mutation, sequencing of the mutation retrieved from in-house service and the generated mutation in the CaR protein sequence.  
B) Representative immunoblot staining against i) Total-CaR and ii) β-actin.  
C) Transiently transfected HEK-293 cells were exposed to increasing Ca^{2+}_o (0.5-10 mM), changes in Ca^{2+}_i were measured (Fura2 ratio). Data was normalised against mean maximal WT response. N=6.
<table>
<thead>
<tr>
<th>CaR Mutant</th>
<th>EC$_{50}$ ± SEM (mM)</th>
<th>Maximum Response (%) ± SEM</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type CaR</td>
<td>3.0 ± 0.1</td>
<td>100 ± 10</td>
<td>23</td>
</tr>
<tr>
<td>Ser-899-Ala</td>
<td>3.0 ± 0.1</td>
<td>106 ± 15</td>
<td>9</td>
</tr>
<tr>
<td>Ser-899-Asp</td>
<td>2.6 ± 0.1 *</td>
<td>93 ± 9</td>
<td>12</td>
</tr>
<tr>
<td>Ser-900-Ala</td>
<td>3.2 ± 0.2</td>
<td>99 ± 7</td>
<td>8</td>
</tr>
<tr>
<td>Ser-900-Asp</td>
<td>3.1 ± 0.2</td>
<td>111 ± 12</td>
<td>9</td>
</tr>
<tr>
<td>Ser-899/900-Ala</td>
<td>2.6 ± 0.2 *</td>
<td>100 ± 8</td>
<td>10</td>
</tr>
<tr>
<td>Ser-899/900-Asp</td>
<td>2.6 ± 0.1 **</td>
<td>100 ± 10</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 4.1 EC$_{50}$ and maximal response by PKA mutants. Mutants transiently expressed in HEK293 cells were exposed to increasing Ca$^{2+}_o$ (0.5–10 mM) and normalised against average maximal WT response. Mutation of S899D, S899-900A, and S899-900D had significantly reduced EC$_{50}$s (*P<0.05, **P<0.01). No mutants had significantly altered maximal responses.

4.4.6 Effect of forskolin on Ca$^{2+}_o$–induced Ca$^{2+}_i$ mobilisation in CaR mutants lacking the PKA sites Ser-899 and Ser-900.

Thus, having established that CaR mutants lacking the PKA sites Ser-899 and Ser-900 still elicit robust Ca$^{2+}_i$ mobilisation in response to elevated Ca$^{2+}_o$ concentration, the effect of forskolin on these responses was then tested. The CaR$^{S900A}$ and CaR$^{S900D}$ single mutants were not specifically tested as these exhibited wild-type behaviour (Table 4.1) in response to Ca$^{2+}_o$, plus the CaR$^{S899A/S900A}$ and CaR$^{S899D/S900D}$ double mutants were both tested and which contain the same Ser-900 mutations anyway. The objective of the experiments was to determine whether deletion of one or both sites could impair the stimulatory response to forskolin which would tend to indicate direct PKA-mediated phosphorylation of the CaR ICD having a functional effect on Ca$^{2+}_i$ mobilisation, as PKC does.

Therefore, the four remaining CaR PKA-mutants (CaR$^{S899A}$, CaR$^{S899D}$, CaR$^{S899A/S900A}$ and CaR$^{S899D/S900D}$) were again transiently transfected into wild-type HEK-293 cells and then exposed to threshold Ca$^{2+}_o$ concentration and finally cotreated with forskolin (10µM). However, the ability of forskolin to potentiate high Ca$^{2+}_o$–induced Ca$^{2+}_i$ mobilisation in wild-type CaR was unaltered in any of the PKA site mutants examined (p>0.05, N≥4; Figure 4.24).

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Figure 4.24. Effect of forskolin treatment in subthreshold Ca$^{2+}$. Panel A) Summary of the change in AUC/min for WT and transiently transfected with CaR$^{S899A}$, CaR$^{S899D}$, CaR$^{S899A/S900A}$, and CaR$^{S899D/S900D}$. There is no significant difference following forskolin treatment. Panels B, C, D and E representative traces of CaR$^{S899A}$, CaR$^{S899D}$, CaR$^{S899A/S900A}$ and CaR$^{S899D/S900D}$ respectively showing single cells (orange and purple) and ‘global’ response (black) to subthreshold Ca$^{2+}$ and forskolin (10 µM) denoted by black bar (N≥4).
4.5 Discussion.

The interaction between intracellular cAMP and CaR signalling was studied using a heterologous expression system, namely CaR-HEK cells. Increasing cytosolic cAMP enhanced intracellular calcium mobilisation and permitted CaR signalling in extracellular calcium concentrations that fail to elicit Ca$^{2+}$i mobilisation i.e. “subthreshold” concentrations. While Gerbino et al had previously shown that cAMP-elevating compounds could increase Ca$^{2+}$i oscillation frequency in response to CaR activation, here I have shown for the first time that cAMP elevation can also lower the Ca$^{2+}$o threshold for CaR-induced Ca$^{2+}$i mobilisation and also that forskolin actually lowers the EC$_{50}$ for Ca$^{2+}$o overall. Furthermore, I provide several lines of evidence casting doubt on the involvement of PKA itself in the stimulatory action of cAMP on CaR signalling and thus will describe possible alternative mechanisms of action.

4.5.1 Use of HEK-293 cells as a suitable model of CaR signalling

Regarding the use of CaR-transfected HEK cells in the current study it should be noted that CaR-HEKs represent the most commonly used model of human CaR structure, pharmacology and signalling in the literature. At present we lack a suitable parathyroid culture model that would have permitted the repetition of many of the current experiments in more physiologically-relevant cells. Although we can in this laboratory obtain bovine parathyroid cells as a by-product of the meat industry, the cell yield is currently too low for biochemical experiments. Also the bovine CaR lacks the PKA site(s) present in human CaR and thus is less suitable for these experiments. Further limitations for bovine PT experiments include variability of the preparation, downregulation of the CaR, contamination with non-chief cells (e.g. fibroblasts) and resistance to transfection.

In contrast, none of these issues apply to CaR-HEK cells. Furthermore, PT and CaR-HEK cells both exhibit similar responses to CaR agonists including PLC-mediated IP$_3$ formation and (oscillatory) Ca$^{2+}$i mobilisation, ERK activation, suppression of cAMP accumulation and the absence of membrane excitability (Gerbino et al, 2005; Kifor et al, 2001; McCormick et al, 2010). In addition, PKC activators and PP2A inhibitors promote CaR$^{T888}$ phosphorylation and suppress CaR-induced Ca$^{2+}$i mobilisation in both cell types. Nevertheless, while the current study sheds light on certain aspects of CaR signalling it will of course be necessary to then develop this
work in parathyroid cells and in humans, as done recently by this laboratory regarding CaR phosphorylation in two studies I have contributed to (Lazarus et al., 2011; McCormick et al., 2010).

4.5.2 The involvement of cAMP and PKC signalling in parathyroid physiology

Mutation of several PKC phosphorylation sites within the CaR ICD, particularly Thr-888, modulates CaR signalling (Davies et al., 2007) and ultimately PTH secretion (Lazarus et al., 2011); however, in vivo it is an increase in cAMP which drives PTH release in the parathyroid gland (Brown & MacLeod, 2001). Indeed, before the CaR was discovered it was noted that activation of endogenously-expressed Gs-linked receptors such as with isoproterenol, dopamine or PGE2, stimulated PTH secretion in bovine parathyroid cells (Brown et al., 1977a; Brown et al., 1977b; Gardner et al., 1978) and that this could be mimicked using the cAMP analogue dibutyryl cAMP (Shoback et al., 1984).

In contrast, activation of Gq/11 leads to Ca2+ mobilisation, which can be detected using Ca2+-sensitive dyes such as FURA2, and is associated in PT cells with suppressed PTH secretion (Shoback et al., 1983; Shoback et al., 1984). Thus, here I investigated the intracellular crosstalk between these two signal pathways to see if they influence each other or remain discrete. In CaR-HEK cells, exposure to moderate Ca2+ concentration elicits CaR-mediated activation and the release of GTP-bound Gαq/11 which recruits PLC (Ward, 2004), in turn hydrolysing PIP2 to IP3 and DAG. The IP3 then binds to IP3R on the ER causing Ca2+ mobilisation (Ward, 2004). DAG and Ca2+ together activate conventional PKCs, which can then phosphorylate the CaR at a number of sites, including Thr-888, which is the primary site leading to inhibition of the receptor (Bai et al., 1998b; Davies et al., 2007). We hypothesise that phosphorylation triggers the activation of a protein phosphatase (most likely PP2A) to then dephosphorylate the receptor, allowing the activation of Gq/11 again (Davies et al., 2007). This proposed cycle of phosphorylation-dephosphorylation could explain the establishment and maintenance of oscillatory Ca2+ mobilisation. Higher agonist concentrations preferentially stimulate the PP2A action causing the CaR to be in a greater state of dephosphorylation thus permitting sustained Ca2+ mobilisation (McCormick, 2008). Gerbino et al reported that increasing cAMP, with isoproterenol, forskolin or VIP, enhanced Ca2+ oscillations and transition to sustained responses (Gerbino et al., 2005). Indeed, data reported here supports these findings in the first instance; namely that increasing cAMP...
enhances intracellular calcium mobilisation, in moderate extracellular calcium (Figure 4.1).

4.5.3 Effect of cAMP on the activation threshold for CaR-mediated Ca\(^{2+}\) mobilisation

It is interesting that the Ca\(^{2+}\)o concentration-effect curve for (G\(_{12/13}\)-mediated) actin polymerisation and membrane ruffling in CaR-HEK cells is left-shifted with respect to (G\(_{q/11}\)-mediated) Ca\(^{2+}\)i mobilisation (EC\(_{50}\), 2.2 mM versus 3.1 mM respectively) (Davey et al, 2012; Gibbons et al, 2012). Significantly, 1.8 mM Ca\(^{2+}\)o which is generally insufficient to elicit Ca\(^{2+}\)i mobilisation or ERK activation in CaR-HEK cells, is in fact able to induce actin polymerisation and other unpublished data from this laboratory and also able to lower cAMP levels (Conigrave et al, 2012; Davies et al, 2006). This suggests that instead of being inactive at ≈1.8 mM Ca\(^{2+}\)o, the CaR is actually active but yet somehow specifically disabled from mobilising Ca\(^{2+}\)i. The current hypothesis is that CaR\(^{T888}\) phosphorylation at these relatively low Ca\(^{2+}\)o concentrations prevents CaR-induced Ca\(^{2+}\)i mobilisation despite the receptor already coupling to actin polymerisation at these Ca\(^{2+}\)o concentrations (Bai et al, 1998b; Davies et al, 2007; McCormick et al, 2010). In support of this, chronic phorbol ester pretreatment, which downregulates PKC, also leftward shifts the Ca\(^{2+}\)o concentration-effect curve (EC\(_{50}\), 2.2 mM vs 3.4 mM control; (Davies et al, 2007). Interestingly, CaR\(^{T888}\) phosphorylation is observed in 1.8 mM Ca\(^{2+}\)o and appears maximal in 2 – 2.5 mM Ca\(^{2+}\)o, i.e. concentrations close to the threshold for Ca\(^{2+}\)i mobilisation (McCormick et al, 2010). It was also interesting that in ≈1.6 mM Ca\(^{2+}\)o little or no Ca\(^{2+}\)i mobilisation was observed and yet Ca\(^{2+}\)i oscillations could be initiated by increasing cAMP levels (Figures 4.2). We would hypothesise therefore that the Ca\(^{2+}\)o threshold for mobilising Ca\(^{2+}\)i is increased by PKC-mediated phosphorylation on CaR\(^{T888}\) but is somehow lowered by raising intracellular cAMP levels. That is, Figures 4.2, 4.3, 4.7, 4.10 and 4.12 show that increasing cAMP, with either forskolin (but not its inactive analogue 1,9-dideoxy FSK), IBMX, pertussis toxin or isoproterenol, allowed Ca\(^{2+}\)i mobilisation at lower Ca\(^{2+}\)o concentrations. Indeed, forskolin was shown to elicit a leftward-shift in the Ca\(^{2+}\)o concentration-effect curve.

Having demonstrated in principle that cAMP lowers the threshold for CaR-induced Ca\(^{2+}\)i mobilisation, it was then necessary to determine the mechanism of action of the cAMP. Firstly, we determined that the stimulatory effect of cAMP also applied to
CaR-induced ERK activation (Figure 4.8). Specifically, it was shown that 3 mM Ca\textsuperscript{2+}, which does not normally elicit significant ERK phosphorylation under control conditions, did elicit ERK activation in the presence of forskolin. Kifor et al was first to show that CaR activation elicits agonist concentration-dependent ERK phosphorylation / activation (Kifor et al, 2001) and further revealed that the effect can be partially inhibited by the PIP\textsubscript{2}-PLC inhibitor U73122. Therefore, it is possible that ERK phosphorylation is merely downstream of PLC-mediated Ca\textsuperscript{2+}\textsubscript{i} mobilisation and thus that cAMP-elicited increases in CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation alone may be sufficient to explain the increased ERK activation. Nevertheless, although not investigated further, the effect of forskolin on ERK phosphorylation did at least confirm that other CaR-mediated signals could be cAMP-modulated.

4.5.4 Identification of possible cyclic AMP targets in lowering the agonist threshold for CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation

The second messenger, cAMP has a plethora of regulatory and functional actions, including regulation of gene transcription, cell proliferation, differentiation and cell death (Antoni, 2012). With regards to GPCR signalling, cyclic AMP might influence Ca\textsuperscript{2+}\textsubscript{i} concentrations via PKA-mediated phosphorylation of IP\textsubscript{3} receptors and/or of the plasma membrane Ca\textsuperscript{2+}-ATPase PMCA (Bruce et al, 2002). Alternatively, the CaR also has two putative PKA sites (CaR\textsuperscript{S899} and CaR\textsuperscript{S900}), whose phosphorylation / dephosphorylation could theoretically influence receptor responsiveness or even membrane localisation (Garrett et al, 1995). Another issue to consider is that the CaR itself couples to G\textsubscript{i/o} in a number of different cell lines and tissues (Chang et al, 1998; Fitzpatrick et al, 1986a) and therefore will tend to lower cAMP levels as it becomes more active, complicating further our understanding of the interaction between cAMP and CaR signalling. As such it was necessary to systematically investigate these various possibilities so as to reveal the likely cause of cAMP-induced CaR potentiation.

4.5.5 Effect of pertussis toxin on CaR-mediated Ca\textsuperscript{2+}\textsubscript{i} mobilisation

The pertussis-sensitive heterotrimeric G-protein G\textsubscript{i/o} suppresses AC action to lower cAMP production and this has been specifically shown for CaR in parathyroid chief cells and CaR-HEK cells (Brown et al, 1990; Fitzpatrick et al, 1986b). Gerbino et al
showed that pertussis treatment reversed spermine-mediated inhibition of PGE
2-cAMP increase (Gerbino et al, 2005). However, not all of the response was G
i/o-mediated as spermine was still capable of partially inhibiting cAMP increase following the pertussis treatment. This inferred that part of the response was likely mediated via a Ca
2+-i-sensitive AC such as AC 5 or 6.

In the current study, overnight pertussis toxin pretreatment lowered the Ca
2+o threshold for Ca
2+i mobilisation though it appeared to do so more via increased efficacy given the significantly increased Ca
2+o maximal responsiveness but unchanged EC
50 (Figure 4.10). In either case, removing G
i/o, enhances Ca
2+i mobilisation at threshold Ca
2+o concentrations in a manner broadly similar to the positive effect of forskolin. Therefore, regardless of whether the elevated cAMP resulted from i) forskolin- or isoproterenol-induced Gas activation, ii) IBMX-suppression of cAMP breakdown or iii) PTx-attenuation of Gαi/o signalling, the simplest explanation for these data is that higher intracellular cAMP levels somehow ‘prime’ the receptor for Ca
2+i mobilisation and/or release it from a prevailing inhibitory signal.

In discussing the use of the various cAMP-altering compounds in this study, it should be noted that while I did not directly assay cAMP levels, Conigrave and co-workers have recently employed a fluorescent cAMP reporter for this purpose. They demonstrated CaR-induced suppression of cAMP in CaR-HEK cells and forskolin-induced elevation of cAMP levels using conditions almost identical to those used here and similar results were also achieved by Gerbino and coworkers using a PKA-based cAMP FRET reporter (Davey et al, 2012; Gerbino et al, 2005). Furthermore, I was able to demonstrate PKA action functionally using an antibody which detects phosphorylation of PKA consensus sites (known as a motif-specific PKA substrate phosphoantibody). With this, I demonstrated significantly increased PKA substrate phosphorylation in response to forskolin cotreatment following incubation in 1.8 mM and 3 mM Ca
2+o. These observations confirmed that forskolin treatment does indeed result in PKA activation but also lead us to consider whether the CaR itself may be a substrate for PKA.

With regards the increase in maximal Ca
2+i response elicited by high Ca
2+o following PTx pretreatment (an observation that I could not find reported elsewhere previously) there are a number of possible explanations for this. Firstly, this apparent increase in CaR efficacy could indicate increased CaR membrane expression (see discussion of “ADIS” in Section 4.5.10), or to increased IP
3R activity
permitting higher maximal Ca\(^{2+}\) mobilisation per se, or even to altered G-protein stoichiometry. That is, assuming that the pleiotropic CaR is normally coupled to a mix of G\(_{i/o}\), G\(_{q/11}\) and G\(_{12/13}\) proteins, then removal of functional G\(_{i/o}\) proteins by PTx could permit greater coupling of the CaRs to the remaining G\(_{q/11}\) (and G\(_{12/13}\)) proteins and thus to heightened Ca\(^{2+}\) mobilisation (and cytoskeletal changes). However this possibility was not investigated further.

4.5.6 Effect of endogenous stimulation of cAMP levels and the potential physiological significance of this effect

It has been shown previously that cAMP can be increased in parathyroid cells and CaR-HEK cells using agonists for receptors expressed endogenously in those cells (Brown et al., 1977a; Brown et al., 1977b; Gardner et al., 1978). Forskolin, PTx and IBMX elicit general, non-physiological increases in cAMP levels. Therefore, I also employed isoproterenol to determine whether activation of endogenous G\(_s\)-linked receptors could also elicit a similar reduction in the Ca\(^{2+}\)\(_o\) threshold for Ca\(^{2+}\)\(_i\) mobilisation, as this would imply that signal crosstalk could actually influence CaR signalling in vivo. Gene microarray identified approximately 16,000 genes expressed in the bovine parathyroid gland; parathyroid hormone and the CaR were ranked 6th and 570th respectively (Table 4.2 and 4.4), tending to validate the microarray ranking as an initial indicator of the likelihood of robust gene expression in the cells. Also indentified in the top 15,000 are a number of G\(_s\)-coupled receptors including histamine, nicotinic, adrenergic, dopamine and prostaglandin receptors. Beta-adrenergic receptors couple to G\(_s\) to activate AC and increase cAMP but do not activate the G\(_{q/11}\) pathway. The \(\beta\)-adrenergic receptor agonist, isoproterenol, increased Ca\(^{2+}\) mobilisation in CaR-HEKs exposed to threshold concentrations of Ca\(^{2+}\)\(_o\) (Figure 4.12). Despite the appearance of isoproterenol-induced Ca\(^{2+}\) mobilisation having occurred, cotreatment with the calcilytic, NPS-2143, ablated the response demonstrating that the G\(_{q/11}\) stimulation was entirely CaR-mediated but that the activated \(\beta 2\)R had somehow lowered the threshold of Ca\(^{2+}\)\(_o\) for CaR-induced Ca\(^{2+}\) mobilisation. A similar effect was observed when the experiment was repeated in cells pretreated with pertussis toxin, possibly due to the removal of the antagonistic G\(_{i/o}\) effect (Figure 4.13). The potential significance of these observations is that in vivo, where Ca\(^{2+}\)\(_o\) concentrations are already partially activating for the CaR, ligands for G\(_s\)-linked receptors that increase cAMP may also be able to modulate Ca\(^{2+}\)\(_i\) mobilisation and CaR activation. That is, the CaR appears able to
integrate intracellular signalling between Ca\textsuperscript{2+} and cAMP concentrations. This could even represent a novel coincidence detector with modest Ca\textsuperscript{2+} concentrations combining with high intracellular cAMP levels from other receptors, to produce heightened CaR-induced Ca\textsuperscript{2+} mobilisation. In fact, given the physiological interplay between cAMP and Ca\textsuperscript{2+} concentrations in PTH secretion, it will be interesting to investigate whether similar observations can be made in cells expressing the CaR endogenously such as PT cells. It is also worth noting that most physiological experiments are conducted under the simplest conditions and with the fewest variables, however in vivo, cells are required to integrate multiple inputs at the same time. This model therefore represents one possible way of investigating such signal integration further.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene Title</th>
<th>Mean</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
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<td>6</td>
</tr>
<tr>
<td>Caveolin1</td>
<td>Caveolin 1 22kDa</td>
<td>5412</td>
<td>416</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
<td>3521</td>
<td>912</td>
</tr>
<tr>
<td>ARPP19</td>
<td>cAMP-regulated phosphoprotein 19kDa</td>
<td>2890</td>
<td>1230</td>
</tr>
<tr>
<td>VEGFB</td>
<td>Vascular endothelial growth factor B</td>
<td>1186</td>
<td>3555</td>
</tr>
<tr>
<td>Filamin-A</td>
<td>Filamin A alpha</td>
<td>727</td>
<td>5287</td>
</tr>
<tr>
<td>MEN1</td>
<td>Multiple endocrine neoplasia I</td>
<td>259</td>
<td>9342</td>
</tr>
<tr>
<td>β-Arrestin1</td>
<td>Arrestin beta 1</td>
<td>114</td>
<td>12181</td>
</tr>
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</table>

Table 4.2. Bovine parathyroid gland microarray data – expressed housekeeping proteins. mRNA was extracted from two bovine parathyroid glands and underwent microarray analysis (see section 2.8.). Mean demonstrates the average signal from 2 sample. Rank of the top 16000 genes detected.
### Table 4.3. Bovine parathyroid gland microarray data – expressed signal related proteins.

mRNA was extracted from two bovine parathyroid glands and underwent microarray analysis (see section 2.8.). ‘Mean’ is the average signal from 2 samples. Rank of the top 16000 genes detected.

<table>
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<tr>
<th>Protein</th>
<th>Gene Title</th>
<th>Mean</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA</td>
<td>cAMP Protein kinase regulatory, type I, alpha</td>
<td>8496</td>
<td>160</td>
</tr>
<tr>
<td>PP2β</td>
<td>Protein phosphatase 2 catalytic subunit beta</td>
<td>4848</td>
<td>528</td>
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<tr>
<td>EPAC12</td>
<td>Rho guanine nucleotide exchange factor 12</td>
<td>4770</td>
<td>540</td>
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<td>ROCK1</td>
<td>Rho-associated coiled-coil containing protein kinase 1</td>
<td>3623</td>
<td>868</td>
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<tr>
<td>PKD3</td>
<td>Protein kinase D3</td>
<td>3227</td>
<td>1045</td>
</tr>
<tr>
<td>PP1A</td>
<td>Mg2+/Mn2+ dependent protein phosphatase 1A</td>
<td>2499</td>
<td>1508</td>
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<td>EPAC3</td>
<td>Rho guanine nucleotide exchange factor (GEF)3</td>
<td>1165</td>
<td>3612</td>
</tr>
<tr>
<td>PP1A</td>
<td>Protein phosphatase 1 catalytic subunit alpha</td>
<td>924.</td>
<td>4393</td>
</tr>
<tr>
<td>EPAC2</td>
<td>Rho/Rac guanine nucleotide exchange factor 2</td>
<td>908</td>
<td>4466</td>
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<tr>
<td>PLCβ4</td>
<td>Phospholipase C beta 4</td>
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<td>4582</td>
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<td>Protein phosphatase 6 catalytic subunit</td>
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<td>6282</td>
</tr>
<tr>
<td>PKCβ</td>
<td>Protein kinase C beta</td>
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</tr>
<tr>
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<td>Protein kinase C eta</td>
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<td>Adenylate cyclase 6</td>
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<td>PKCζ</td>
<td>Protein kinase C zeta</td>
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<td>7523</td>
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<tr>
<td>PDE4B</td>
<td>cAMP-specific Phosphodiesterase 4B</td>
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<td>7910</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase catalytic alpha</td>
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<td>8673</td>
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<td>PDE12</td>
<td>Phosphodiesterase 12</td>
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<td>9094</td>
</tr>
<tr>
<td>PLCδ1</td>
<td>Phospholipase C delta 1</td>
<td>185</td>
<td>10635</td>
</tr>
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<td>10638</td>
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<td>PP4</td>
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<td>AC 8</td>
<td>Adenylate cyclase 8</td>
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<td>13923</td>
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<td>Calmodulin-dependent phosphodiesterase 1B</td>
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<td>14199</td>
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Table 4.4. Table of selected receptors from bovine microarray data. mRNA was extracted from two bovine parathyroid glands and underwent microarray analysis (see section 2.8.). Mean demonstrates the average signal from 2 sample. Rank of the top 16000 genes detected.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene_Title</th>
<th>Mean</th>
<th>Rank</th>
</tr>
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<tbody>
<tr>
<td>CaR</td>
<td>Calcium-Sensing Receptor</td>
<td>4648</td>
<td>570</td>
</tr>
<tr>
<td>TGFR</td>
<td>Transforming growth factor beta receptor II</td>
<td>2598</td>
<td>1439</td>
</tr>
<tr>
<td>DopamineR</td>
<td>Dopamine receptor D1</td>
<td>1753</td>
<td>2364</td>
</tr>
<tr>
<td>γGABARa</td>
<td>Gamma-aminobutyric_acid (GABA)A receptor alpha</td>
<td>934</td>
<td>4354</td>
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<tr>
<td>PGER4</td>
<td>Prostaglandin E receptor 4(EP4)</td>
<td>834</td>
<td>4795</td>
</tr>
<tr>
<td>ProlactinR</td>
<td>Prolactin receptor</td>
<td>714</td>
<td>5359</td>
</tr>
<tr>
<td>IP3R2</td>
<td>Inositol 1,4,5-triphosphate receptor, type_2</td>
<td>620</td>
<td>5909</td>
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<tr>
<td>IL17Ra</td>
<td>Interleukin 17 receptor A</td>
<td>591</td>
<td>6095</td>
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<tr>
<td>β-AdrenergicR</td>
<td>Beta-2 Adrenergic receptor</td>
<td>502</td>
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<tr>
<td>P2YR</td>
<td>G-protein coupled purinergic receptor P2 10</td>
<td>495</td>
<td>6772</td>
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<td>P2XR</td>
<td>Ligand-gated ion channel purinergic receptor P2X</td>
<td>342</td>
<td>8259</td>
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<tr>
<td>PGFR2</td>
<td>Prostaglandin F2 receptor negative_regulator</td>
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<td>8308</td>
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<td>8520</td>
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<tr>
<td>EndothelinR</td>
<td>Endothelin receptor type A</td>
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<td>9418</td>
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<td>ILR6</td>
<td>Interleukin 6 receptor</td>
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<td>9884</td>
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<td>ILR1</td>
<td>Interleukin 1 receptor type I</td>
<td>220</td>
<td>9978</td>
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<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor 4</td>
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<td>10296</td>
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<tr>
<td>δ-NicotinicR</td>
<td>Cholinergic receptor nicotinic delta</td>
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<td>10975</td>
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<tr>
<td>H1</td>
<td>Histamine receptor H1</td>
<td>128</td>
<td>11839</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
<td>120</td>
<td>12031</td>
</tr>
<tr>
<td>GHR</td>
<td>Growth hormone receptor</td>
<td>110</td>
<td>12281</td>
</tr>
<tr>
<td>mGLUR7</td>
<td>Metabotropic glutamate receptor 7</td>
<td>92</td>
<td>12735</td>
</tr>
<tr>
<td>P2YR</td>
<td>G-protein coupled purinergic receptor_P2Y 13</td>
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<tr>
<td>GPR5A</td>
<td>G protein-coupled receptor familyC group 5 memberA</td>
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<td>13582</td>
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<tr>
<td>PGFR</td>
<td>Prostaglandin F receptor (FP)</td>
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<td>13840</td>
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<td>PTHR</td>
<td>Parathyroid_Hormone_1_Receptor</td>
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<td>14192</td>
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</table>

4.5.7 Effect of PKA inhibition on $Ca^{2+}_o$ induced $Ca^{2+}_i$ mobilisation

There are a number of possible intracellular targets for cAMP, but the most obvious of these is PKA. Thus, I next investigated whether the stimulatory effect of cAMP occurred via PKA activation. The effect of inhibiting PKA was determined using two PKA inhibitors, H-89 and KT5720. Neither inhibitor decreased the effect of forskolin on $Ca^{2+}_i$ mobilisation in either moderate (3 mM) or subthreshold (~1.6 mM) extracellular calcium concentrations. One possible explanation for the cAMP-induced increase in CaR-induced $Ca^{2+}_i$ mobilisation is PKA-mediated phosphorylation of the IP$_3$ receptor whereby PKA sensitises the IP$_3$R to IP$_3$; (Bruce
et al, 2002). However, these data would tend to discount this given that the FSK effect is maintained despite the inhibited PKA activity.

Bosel et al demonstrated that cotreatment of CaR agonists with the PKA inhibitor H89, increased IP metabolism (Bosel et al, 2003). Interestingly, the same group showed that pretreatment with the PKC inhibitor GF109203X, and H89, had a greater effect than treatment with either inhibitor alone. They suggested that the PKC and PKA pathways converge to phosphorylate the receptor, or that the protein kinases act on downstream targets (Bosel et al, 2003). It should also be noted that I observed a basal increase in Ca$^{2+}_i$ (p>0.05 by One-way ANOVA) in the presence of a PKA inhibitor, KT5720 that was not seen with H89 which might suggest non-specific actions of at least one of the compounds. Indeed, the Cohen laboratory have previously reported inhibitor selectivity issues with H89 and KT5720 proving capable of inhibiting other kinases (Davies et al, 2000).

One possible way to delineate downstream cAMP effectors is with the use of cyclic AMP analogues that specifically activate only PKA or EPAC. Previous groups have used cAMP analogues in various CaR studies and in a variety of cell lines to investigate changes in Ca$^{2+}_i$, membrane potential, cAMP and Cl$^-$ flux (Cifuentes & Rojas, 2008; De Jesus Ferreira & Bailly, 1998; Gerbino et al, 2005; Kong et al, 2012). However, in the current study neither acute nor chronic application of PKA- or EPAC-selective cAMP analogues consistently altered Ca$^{2+}_i$ concentrations. Whether I had failed to achieve sufficient cellular accumulation of the cAMP analogues, or they lacked efficacy, could not be easily determined. It was therefore decided that to determine whether PKA modulates CaR signalling by directly phosphorylating the receptor, it would be necessary to mutate the putative PKA sites within the receptor.

4.5.8 Functional consequence of mutating the PKA consensus sites in CaR

Garrett et al identified two putative PKA sites in the human CaR at Ser-899 and Ser-900. These consecutive residues appear part of a potential ER-retention sequence, namely R-K-R-S-S (Garrett et al, 1995).

However, mutation of CaR$^{S899}$ to a non-phosphorylatable alanine residue had no apparent effect on the ability of the receptor to mobilise Ca$^{2+}_i$ (Figure 4.21). Interestingly, phosphomimetic mutation of the site, CaR$^{S899D}$, did exhibit a significant reduction in the EC$_{50}$ of Ca$^{2+}_o$ for CaR-induced Ca$^{2+}_i$ mobilisation.
Therefore any intracellular signal that can promote CaR\textsuperscript{S899} phosphorylation may indeed be able to increase CaR responsiveness at moderate Ca\textsuperscript{2+}o concentrations. However, since the CaR\textsuperscript{S899A} mutant exhibited wild-type-like behaviour, it can only be concluded that CaR\textsuperscript{S899} phosphorylation does not routinely occur following activation by Ca\textsuperscript{2+}o or that if it does it has no functional consequence. Furthermore, even though CaR\textsuperscript{S899D} did exhibit heightened sensitivity to Ca\textsuperscript{2+}o, neither mutant inhibited the stimulatory effect of forskolin (Figure 4.22) suggesting that it has no relevance to the current study.

With regards CaR\textsuperscript{S900}, neither mutation of this residue to either alanine or aspartate had any functional effect on receptor responsiveness to Ca\textsuperscript{2+}o (Figure 4.21 and 4.22), or indeed to forskolin (Figure 4.24).

Interestingly, double mutation of both putative PKA sites to alanine did increase CaR sensitivity / responsiveness relative to wild-type CaR (discussed further in 4.5.10), however it failed to attenuate the forskolin response and thus tends to rule out a role for PKA-mediated CaR phosphorylation following forskolin treatment (Figure 4.24). Similarly, CaR\textsuperscript{S899D/S900D} also exhibited increased CaR responsiveness relative to wild-type, with the effect no greater than for CaR\textsuperscript{S899D} alone but most significantly failed to attenuate forskolin responsiveness.

Previous work by this laboratory and others has shown that phosphomimetic mutation of Thr-888 (to aspartate or glutamate) inhibits receptor responsiveness while phospho-null mutation (e.g. to alanine or methionine) causes a leftward shift in CaR sensitivity (Bai et al, 1998b; McCormick et al, 2010) and thus to suppressed PTH secretion in man (Lazarus et al, 2011). Therefore, the techniques employed in the current study were capable of detecting changes in receptor responsiveness had they occurred, however, the behaviour of CaR\textsuperscript{S899A} and CaR\textsuperscript{S900A} in response to Ca\textsuperscript{2+}o was entirely unchanged from wild-type CaR behaviour. It should also be noted however that while Garrett et al predicted there being only two PKA sites in CaR, at Ser-899 and Ser-900, this does not preclude the possibility of there being further PKA sites elsewhere. Indeed, in the current laboratory we have identified an additional PKC site in CaR (unpublished observation) not originally predicted by Garrett et al (Garrett et al, 1995). That said, only a few of the remaining serine / threonine residues in the ICD are downstream of an arginine residue and thus the prospect of there being an alternative / additional PKA site in CaR appears relatively limited.
4.5.9 Possible mechanism of cAMP influence in CaR signalling

The data presented here demonstrates for the first time that increasing intracellular cAMP levels enables CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation in sub-threshold Ca\textsuperscript{2+}\textsubscript{o} concentrations. The working hypothesis is that there are low-to-moderate Ca\textsuperscript{2+}\textsubscript{o} concentrations at which the CaR is able to signal via G\textsubscript{12/13} and G\textsubscript{i/o}, and yet unable to elicit G\textsubscript{q/11}-mediated Ca\textsuperscript{2+}\textsubscript{i} mobilisation. There appear two possible explanations for this, the first is that PKC-mediated CaR\textsuperscript{T888} phosphorylation raises the Ca\textsuperscript{2+}\textsubscript{o} threshold for Ca\textsuperscript{2+}\textsubscript{i} mobilisation (Lazarus \textit{et al}, 2011; McCormick \textit{et al}, 2010). The second possibility is that G\textsubscript{i/o} activation decreases basal cAMP levels thus removing any potential stimulatory benefit of cAMP. It should be noted that these two possibilities are by no means mutually exclusive and in fact the latter could even potentiate the effect of the former. To clarify this issue it should be noted that I have shown experimentally that there are two ways to overcome the inhibition of Ca\textsuperscript{2+}\textsubscript{i} mobilisation described above. The first is to further increase the Ca\textsuperscript{2+}\textsubscript{o} concentration which will cause PP2A-mediated dephosphorylation of the CaR\textsuperscript{T888} leading to Ca\textsuperscript{2+}\textsubscript{i} mobilisation. The second is to increase intracellular cAMP levels to sensitise the CaR to the existing Ca\textsuperscript{2+}\textsubscript{o} concentration, possibly by activation of a cAMP-sensitive phosphatase leading again, to CaR\textsuperscript{T888} dephosphorylation. Further experiments will be necessary to determine the effect of forskolin on the phosphorylation state of CaR\textsuperscript{T888} in threshold Ca\textsuperscript{2+}\textsubscript{o} concentrations. If forskolin does elicit CaR\textsuperscript{T888} dephosphorylation, this could indicate that it is acting via a cAMP-sensitive phosphatase. This could be beneficial in tissues that have high basal levels of cAMP. It might even be noted that the set-point for PTH secretion in \textit{vivo}, is lower (≈1.2-1.4 mM) than for CaR’s EC\textsubscript{50} \textit{in vitro} (3.0 mM) in HEK-293 cells. With the PT cells expressing histamine, adrenergic, dopamine and prostanoid receptors there is ample opportunity for these cells to elevate cAMP levels close to the CaR. Therefore, to address this further it will be necessary to assess the effect of cAMP-elevating agents on CaR\textsuperscript{T888} phosphorylation (McCormick \textit{et al}, 2010).

It is also noteworthy that a number of other cAMP-mediated proteins were detected in the microarray; including adenylate cyclases 3 and 8 which are stimulated by cAMP and thus also by PDE action. The transcription factors cAMP-responsive element binding (CREB) and cAMP-responsive element modulator (CREM) are both expressed in CaR-HEK (unpublished microarray data). In this regard, Avlani \textit{et al} recently reported that although CaR activation decreases cAMP, CaR dose-dependently stimulates CREB phosphorylation via a PI-PLC and PKC dependent manner (Avlani \textit{et al}, 2013).
Regarding possible physiological interactions between CaR and cAMP signalling it should be noted that it has recently been shown in juxtaglomerular cells, that CaR elicits decreased cAMP accumulation, and, inhibits renin secretion, in a PLC-dependent, G\textsubscript{i}-independent manner (Ortiz-Capisano \textit{et al}, 2013). Cyclic AMP drives renin secretion via PLC; activation of the CaR with Ca\textsuperscript{2+}\textsubscript{o} stimulates Ca\textsuperscript{2+} mobilisation, which inhibits cAMP synthesis via Ca\textsuperscript{2+} sensitive-ACs 5 and 6, inhibiting further renin secretion (Grünberger \textit{et al}, 2006; Ortiz-Capisano \textit{et al}, 2013). In parathyroid cells, cAMP stimulates PTH, via a currently unknown pathway. Thus, here we have shown that cAMP also stimulates CaR activation, which could feedback, limiting further cAMP via Ca\textsuperscript{2+}-sensitive ACs.

\subsection*{4.5.10 Relevance of CaR\textsuperscript{S899A} functional data for the concept of agonist-driven insertional signalling}

Agonist-driven insertional signalling (ADIS) is a theory proposed by the Breitwieser group as an alternative or complementary explanation for high cooperativity and lack of desensitisation demonstrated by CaR (Breitwieser; Grant \textit{et al}, 2011). Simply put, activation of CaR with extracellular agonists drives further CaR trafficking to the plasma membrane with the possibility of enhanced signalling at the top of the Ca\textsuperscript{2+}\textsubscript{o} concentration-effect curve.

Some CaR gain- or loss-of-function mutations are known to affect membrane expression, and decreased membrane expression is associated with FHH and non-calciostatic diseases such as colon cancer, pancreatitis and epilepsy (Breitwieser; Grant \textit{et al}, 2012; Grant \textit{et al}, 2011; Stepanchick \textit{et al}, 2010). Use of CaR-activating calcimimetics can rescue such mutants, increasing their membrane expression (White \textit{et al}, 2009) and implying that agonist exposure will increase membrane expression.

The arginine-rich motif RXR controls endoplasmic reticulum retention in multiple membrane proteins and one such motif is located in the proximal C-terminus of CaR at Arg-886 to Arg-898 (Grant \textit{et al}, 2011; Stepanchick \textit{et al}, 2010). Mutation of this RKR-motif in CaR is known to cause both loss-and gain-of-function mutations, which Stepanchick \textit{et al} hypothesised were due to an alteration in plasma membrane expression (Stepanchick \textit{et al}, 2010).

The RKR-motif is close to the PKC site Ser-892 and putative PKA phosphorylation site Ser-899. Stepanchick \textit{et al} reported that mutation of the PKC site did not affect
membrane expression; whereas the mutant CaR^{S899D} significantly increased membrane expression. CaR^{S899D} was reported to exhibit increased abundance as a mature 160 kDa receptor, although this was not depicted with a loading control. In contrast, I did not observe an apparent difference in 160 kDa, mature CaR^{S899D} expression but did observe increased sensitivity (Figure 4.22). However, as already stated there was no functional effect on CaR responsiveness of mutating CaR^{S899} to alanine.

Breitwieser and co-workers hypothesised that phosphorylation of Ser-899 affected ER retention by influencing the binding of 14-3-3 proteins. 14-3-3 proteins are known to influence ER retention and may act directly or indirectly to influence CaR retention (Arulpragasam et al., 2012). Breitwieser et al show that CaR^{S899D} significantly decreased co-immunoprecipitation between CaR and 14-3-3, compared to WT (Grant et al., 2011; Stepanchick et al., 2011) whereas CaR^{S899A} exhibited significantly increased 14-3-3 co-immunoprecipitation (Grant et al., 2011). This suggests that phosphorylation of Ser-899 decreases interaction between the CaR and 14-3-3 allowing trafficking to the membrane. Using TIRF, Breitwieser and coworkers demonstrated Ca^{2+} concentration-dependent increases in WT CaR plasma membrane expression (Grant et al., 2011). CaR^{S899D} did not exhibit substantial Ca^{2+} dependent increases in PM expression although this might have been because CaR^{S899D} was already exhibiting (near) maximal membrane expression that could not be further heightened by ADIS. Such an explanation might fit with the functional effect for CaR^{S899D} observed in the current study. However, CaR^{S899A} exhibited decreased maximal response to ADIS which was hypothesised to result from increased ER retention (Grant et al., 2011). Breitwieser et al concluded that CaR^{S899} fine-tunes ADIS possibly as one of a number of phosphorylation sites regulating this phenomenon (Breitwieser, 2012). However, in our hands CaR^{S899A} exhibits responsiveness no different to the wild-type CaR. I would argue therefore that since pseudo-phosphorylation of CaR^{S899} did cause a positive functional effect (CaR^{S899D}), then permanently inhibiting that phosphorylation i.e. with CaR^{S899A}, should have elicited the opposite effect, as is the case for CaR^{T888} mutations. Since it did not then the simplest explanation for the current data is that CaR^{S899A} signals perfectly normally even if it alters ADIS and therefore it is possible that while ADIS is an important explanation for how the CaR avoids desensitisation, it may be less important for regulating CaR responsiveness for receptors already on the membrane. It should be noted however that while the responsiveness of CaR^{S899A} and CaR^{S900A} was no different to that for wild-type CaR, double mutation to CaR^{S899A/S900A} did in fact produce a significantly lower EC_{50} for Ca^{2+} on CaR-induced
Ca\textsuperscript{2+} mobilisation. Because of this, one might speculate that provided there is phosphorylation of either PKA site (899 or 900) the receptor is partially inhibited (either functionally or in terms of ADIS) and thus both phospho-sites must be deleted to achieve an increase in CaR signal. However, there would be several arguments against this. The first is that the phosphomimetic mutants CaRS899D and CaR\textsuperscript{S899D/S900D} exhibit increased not decreased sensitivity. And secondly, in pharmacology it is usually understood that EC\textsubscript{50} changes reflect altered agonist binding whereas increased receptor availability instead elevates the E\textsubscript{max} (maximal response) without necessarily altering the sensitivity of individual receptors. In the current study, the PKA site mutations either increase Ca\textsuperscript{2+} agonist potency (lower EC\textsubscript{50}) or have no effect at all, but none of them alter E\textsubscript{max}, casting further doubt on the idea that ADIS, via Ser-899 at least, increases total CaR responsiveness.

Breitwieser \textit{et al} argue that it is not possible to determine if the steep relationship observed in a conventional cumulative concentration-effect curve is due to agonist cooperativity or ADIS (Breitwieser, 2012). This is because increasing the Ca\textsuperscript{2+} concentration in a step-wise manner may also incorporate the effect of ADIS, increasing CaR membrane expression. However, comparing the concentration-effect data for WT and CaR\textsuperscript{S899D} shows that in 2 mM, the first point on the curve, the mutant is significantly more active. This time-point would not allow for ADIS to have effect and therefore we can assume that this mutant is genuinely more functionally active. Thus, if CaR\textsuperscript{S899A} is retained in the ER then given the ADIS hypothesis then we would have expected decreased CaR responsiveness, however, CaR\textsuperscript{S899A} exhibited wild-type-like behaviour.

In order to demonstrate if CaR cooperativity is due to ADIS or not, it would be helpful to compare the Ca\textsuperscript{2+} concentration-dependency of CaR-induced Ca\textsuperscript{2+} mobilisation between cell exposed to cumulative Ca\textsuperscript{2+} concentrations, and, those challenged with elevated Ca\textsuperscript{2+} followed by a return to low Ca\textsuperscript{2+} again following each challenge. That is, the latter method should not be affected by cumulative ADIS although such a protocol is less physiological given that CaR is usually constantly exposed to agonist and such exposures change incrementally as blood calcium levels rise or fall but do not change dramatically. Furthermore if ADIS does increase signalling, then exposing CaR-HEK cells to a single calcium concentration would increase calcium mobilisation over time; however, this has never been observed. The CaR is one of the few GPCRs that does not significantly desensitise and it is clear that the ADIS proposal by Breitwieser \textit{et al} is an important development in
explaining that resistance to desensitisation (Breitwieser, 2012). However, whether ADIS also explains CaR cooperativity remains to be demonstrated.

4.5.11 Summary

In conclusion, the data presented here suggest that the human CaR is subject to previously unrecognised control by cytosolic cAMP. In extracellular calcium concentrations which do not elicit $G_{q/11}$ mediated $Ca^{2+}$ mobilisation, increasing cAMP permits, or augments, $Ca^{2+}$ mobilisation. The results here show that it is unlikely that this is PKA-mediated and thus might be better explained by CaR dephosphorylation by a protein phosphatase.
Chapter 5

General Discussion
5.1 General discussion

The ability of the CaR to sense non-physiological changes in extracellular pH was demonstrated previously by Quinn et al. However, further investigation by this laboratory has revealed that pathophysiological pH changes (± 0.2 unit) can also be detected by CaR (Quinn et al., 2004). An increase in pH potentiates CaR-induced Ca\textsuperscript{2+} mobilisation, ERK phosphorylation and actin polymerisation while a more acidic pH attenuates CaR signalling. Such pathophysiological changes can occur naturally during senescence, due to declining renal function, but develops more markedly in patients with renal failure. Since protons and calcium are subject to potential buffering by phosphate, albumin and carbonate it was important that I first demonstrated that the CaR's pH sensitivity could still be observed in the presence of a physiological concentration of albumin (5%).

Next, I attempted to identify the molecular mediator of CaR pH sensitivity with one or more of the extracellular histidine residues considered to be the most likely site(s) as shown in certain other membrane proteins and based on the pH sensitivity of their ionisable sidechain. However, site-directed mutation of all sixteen extracellular histidines (including at least one double mutation) did not attenuate pH sensitivity in any case. Although some mutations exhibited modest trend changes in pH sensitivity, none of the responses were significantly different from WT behaviour. The free, extracellular cysteine residue was also mutated but this did not alter CaR pH sensitivity either. As a result, I would conclude that clusters of glutamate and aspartate amino acids that form the putative calcium binding regions of the receptor would appear to be the most likely mediators of CaR pH sensitivity.

Next, the relationship between CaR signalling and cytosolic cAMP was investigated. It has previously been noted that in comparison to CaR-induced actin polymerisation / membrane ruffling, the Ca\textsuperscript{2+} concentration-effect relationship for Ca\textsuperscript{2+} mobilisation is right-ward shifted. This suggests that at moderate Ca\textsuperscript{2+} concentrations (1.8 mM in this study) the CaR may be conformationally active and yet uncoupled from the mechanism of Ca\textsuperscript{2+} mobilisation. As a result, releasing the receptor from this inhibition may even contribute to the steepness of the agonist-effect relationship.

I first confirmed the Gerbino et al. observation that agents which increase cAMP concentrations also enhance Ca\textsuperscript{2+} mobilisation in CaR-HEK cells (Gerbino et al., 2005). Next, I determined the consequence of increasing cAMP levels on the threshold Ca\textsuperscript{2+} concentration for Ca\textsuperscript{2+} mobilisation. Increasing cytosolic cAMP...
concentration, with forskolin, isoproterenol, IBMX or pertussis toxin significantly increased CaR-induced Ca^{2+}_i mobilisation, triggering the initiation of oscillations, with forskolin also potentiating CaR-mediated ERK phosphorylation. The potential importance of the isoproterenol observation is that activation of any G_s-linked GPCR in a CaR-expressing cell exposed to a moderate Ca^{2+}_o concentration could also result in altered Ca^{2+}_i mobilisation.

One possible explanation for the positive effect of increasing cytosolic cAMP on Ca^{2+}_i mobilisation is via PKA-mediated phosphorylation of the IP_3R. However, neither of the two PKA inhibitors used was able to attenuate the forskolin response. Furthermore, regarding the possible regulation of receptor function by PKA-mediated phosphorylation on CaR^{S899} or CaR^{S900}, neither mutant overcame the positive effect of forskolin cotreatment on Ca^{2+}_i mobilisation. Thus, the simplest explanation of the current data is that increased cAMP concentration does not elicit its positive effect on CaR-induced Ca^{2+}_i mobilisation via PKA activation and thus it should be next investigated whether instead cAMP somehow stimulates the dephosphorylation of CaR^{T888} which could potentially release the restraint on Ca^{2+}_i mobilisation. However, possible potentiation of IP_3R-mediated Ca^{2+}_i release has not been ruled out by these data.

It should be noted however that phosphomimetic mutation of CaR^{S899} did significantly left-shift the Ca^{2+}_o concentration-effect relationship for Ca^{2+}_i mobilisation which indicates that phosphorylation of this site may alter either function or even membrane localisation of the receptor. That said however, the function of the phospho-null mutant CaR^{S899A}, was equivalent to WT-CaR which suggests that the site does not become phosphorylated as Ca^{2+}_o concentration is increased as otherwise it should have caused a right-shift in the Ca^{2+}_o concentration-effect relationship. If this conclusion is correct, then these data would argue against CaR^{S899} playing a functional role in ADIS. That is, even if CaR^{S899} does contribute to ADIS, it does not alter CaR signalling (Ca^{2+}_i mobilisation in this case) significantly.

In any case, that forskolin-elicited an increase in Ca^{2+}_i mobilisation in both CaR^{S899A} and CaR^{S900A} indicates that these putative PKA sites (in the WT receptor) do not mediate the positive effect of forskolin. It has been hypothesised by Breitwieser and coworkers that phosphorylation of Ser-899 increases membrane expression; this could be supported by data presented here (Grant et al, 2011; Stepanchick et al, 2010). However, there is no functional loss from not phosphorylating this site (S899A), which suggests that this is not functionally relevant. Agonist-driven
insertional signalling (ADIS) proposes a solution for the lack of desensitisation and high degree of cooperativity. Evidence presented here suggests that Ser-899 does not contribute to receptor function and mutation does not alter maximal receptor activation which suggests no alteration to efficacy. Furthermore if ADIS, and specifically S899A, did contribute to cooperativity then mutation would cause a decrease in activation. Breitwieser and coworkers demonstrated that in WT receptor increase Ca\(^{2+}\) increase plasma membrane expression \(\approx 300\) seconds; therefore in a single calcium concentration we would expect to see an increase in calcium mobilisation after this amount of time, however none has ever been observed here (Grant et al, 2011). One explanation which may explain this is that ADIS contributes to the maintenance of ‘signalling’ receptors on the membrane, but not to signal potentiation or cooperativity.

### 5.2 Conclusions

In conclusion, the data presented here have addressed two fundamental areas regarding CaR responsiveness. In the first case, I have shown that none of the CaR’s extracellular histidine or free cysteine residues are responsible for mediating extracellular pH sensitivity and that pathophysiological changes in pH\(_o\) are not compensated for by buffering with albumin. With the eventual precise determination of CaR’s Ca\(^{2+}\) binding sites, it will then become possible to test whether mutation of these residues (or local disruption of these clusters) attenuates the pH\(_o\) sensitivity.

In the second case, I have revealed that increasing intracellular cAMP concentrations lowers the threshold Ca\(^{2+}\) concentration for CaR-induced Ca\(^{2+}\) mobilisation, at least in HEK-293 cells. This raises the question then of whether this effect occurs \textit{in vivo} in cells in which the CaR is expressed endogenously.

By better understanding how cAMP and CaR signalling is integrated, this will help us to determine the mechanism of PTH secretion and its homeostatic control. With the increasing incidence of osteoporosis and kidney disease these issues are not only of biological interest but of increasing clinical importance.
REFERENCES


Bai M, Trivedi S, Lane CR, Yang Y, Quinn SJ, Brown EM (1998b) Protein kinase C phosphorylation of threonine at position 888 in Ca2+o-sensing receptor (CaR) inhibits coupling to Ca2+ store release. *J Biol Chem* **273**: 21267-21275


line: evidence for direct regulation of vitamin D metabolism by calcium. *Endocrinology* **140:** 2027-2034


Bramucci E, Paiardiini A, Bossa F, Pascarella S (2012) PyMod: sequence similarity searches, multiple sequence-structure alignments, and homology modeling within PyMOL. *BMC Bioinformatics* **13 Suppl 4:** S2


Brown EM (1991) Extracellular Ca2+ sensing, regulation of parathyroid cell function, and role of Ca2+ and other ions as extracellular (first) messengers. *Physiol Rev* **71:** 371-411


monophosphate accumulation, and the levels of inositol phosphates in bovine parathyroid cells. *Endocrinology* **127**: 1064-1071


Chow JY, Estrema C, Orneles T, Dong X, Barrett KE, Dong H Calcium-sensing receptor modulates extracellular Ca(2+) entry via TRPC-encoded receptor-operated channels in human aortic smooth muscle cells. *Am J Physiol Cell Physiol* **301:** C461-468


polymorphisms and expression levels in end-stage renal disease patients. Clin Nephrol 72: 114-121


Fitzpatrick LA, Brandi ML, Aurbach GD (1986a) Calcium-controlled secretion is effected through a guanine nucleotide regulatory protein in parathyroid cells. Endocrinology 119: 2700-2703

Fitzpatrick LA, Brandi ML, Aurbach GD (1986b) Control of PTH secretion is mediated through calcium channels and is blocked by pertussis toxin treatment of parathyroid cells. Biochem Biophys Res Commun 138: 960-965


Godwin SL, Soltoff SP (2002) Calcium-sensing receptor-mediated activation of phospholipase C-gamma1 is downstream of phospholipase C-beta and protein kinase C in MC3T3-E1 osteoblasts. Bone 30: 559-566


Mamillapalli R, Wysolmerski J The calcium-sensing receptor couples to Galpha(s) and regulates PTHrP and ACTH secretion in pituitary cells. *J Endocrinol* **204**: 287-297


McCormick W (2008) Characterisation of calcium-sensing receptor signalling and feedback regulation in endogenous expression systems. Doctor of Philosophy (PhD) Thesis, Faculty of Life Sciences, University of Manchester,


Parkash J (2008a) Inflammatory cytokine signaling in insulin producing beta-cells enhances the colocalization correlation coefficient between L-type voltage-dependent calcium channel and calcium-sensing receptor. *Int J Mol Med* **22**: 155-163


Pi M, Hinson TK, Quarles L (1999) Failure to detect the extracellular calcium-sensing receptor (CasR) in human osteoblast cell lines. *J Bone Miner Res* **14**: 1310-1319


Pi M, Spurney RF, Tu Q, Hinson T, Quarles LD (2002) Calcium-sensing receptor activation of rho involves filamin and rho-guanine nucleotide exchange factor. *Endocrinology* **143**: 3830-3838


Ca2+-sensing receptor in normal human gastric mucous epithelial cells. Am J Physiol 277: G662-670


Vassilev PM, Ho-Pao CL, Kanazirska MP, Ye C, Hong K, Seidman CE, Seidman JG, Brown EM (1997) Ca(2+)-sensing receptor (CaR)-mediated activation of K+ channels is blunted in CaR gene-deficient mouse neurons. *Neuroreport* **8**: 1411-1416

polymorphism of calcium-sensing receptor does produce a gain-of-function and predispose to primary hypercalciuria. *Kidney Int* **71**: 1155-1162


Ye C, Rogers K, Bai M, Quinn SJ, Brown EM, Vassilev PM (1996b) Agonists of the Ca(2+)-sensing receptor (CaR) activate nonselective cation channels in HEK293 cells stably transfected with the human CaR. *Biochem Biophys Res Commun* **226**: 572-579


