Signal Crosstalk between Calcium-Sensing and Prostanoid Receptors and its Role in Parathyroid Hormone Secretion

A thesis submitted to The University of Manchester for the degree of

Doctor of Philosophy (PhD)

in the Faculty of Biology, Medicine and Health

2017

Halah Tariq Albar

School of Medical Sciences

Division of Diabetes, Endocrinology and Gastroenterology
Table of Contents

LIST OF FIGURES .................................................................9
LIST OF TABLES .................................................................12
LIST OF ABBREVIATIONS .....................................................13
ABSTRACT .............................................................................17
DECLARATION .........................................................................18
COPYRIGHT STATEMENT .......................................................19
ACKNOWLEDGEMENT ............................................................20
CHAPTER 1 ............................................................................21
Introduction ............................................................................21
  1.1 Introduction .....................................................................22
  1.2 Physiology of Calcium homeostasis ...............................22
  1.3 Calcium distribution between kidney, bone, intestine and extracellular fluid (ECF) ........................................23
    1.3.1 Intestinal calcium absorption ....................................23
    1.3.2 Renal calcium reabsorption .......................................25
    1.3.3 Skeletal mineral storage .............................................26
  1.4 Endocrine control of serum calcium concentration ..........27
    1.4.1 Parathyroid hormone ................................................27
    1.4.2 Vitamin D ..................................................................27
    1.4.3 Calcitonin .................................................................28
  1.5 Parathyroid hormone ......................................................29
    1.5.1 Parathyroid hormone function ...................................29
    1.5.2 Regulation of parathyroid hormone secretion .............30
    1.5.3 Diseases of parathyroid hormone secretory dysfunction: primary and secondary hyperparathyroidism ............31
  1.6 Calcium-sensing receptor ...............................................33
    1.6.1 Structure ..................................................................33
    1.6.2 CaR pharmacology ...................................................35
    1.6.3 Diseases resulting from CaR mutation and dysfunction .................................................................37
    1.6.4 CaR signalling ..........................................................37
    1.6.5 Signal bias and CaR ....................................................39
  1.7 Potential Gαs-coupled GPCRs signalling involved in the regulation of PTH secretion ........................................41
1.7.1 Prostanoid receptors .................................................................................41
  1.7.1.1 Prostanoid synthesis and function .................................................41
  1.7.1.2 Prostanoid receptors signalling ......................................................41
  1.7.1.3 Prostanoid receptors pharmacology ..............................................42
  1.7.1.4 Prostanoids in parathyroid gland ..................................................42
1.7.2 Histamine receptors .................................................................................44
  1.7.2.1 Histamine synthesis and function ..................................................44
  1.7.2.2 Histamine receptors signalling ......................................................44
  1.7.2.3 Histamine signalling in parathyroid gland ....................................45
1.7.3 B-adrenergic receptors ..............................................................................46
1.7.4 Calcitonin receptor-like receptor (CALCRL) ............................................47
1.8 Aims and objectives .....................................................................................47

CHAPTER 2 ...........................................................................................................49
Materials & Methods ..........................................................................................49
  2.1 Materials ..................................................................................................50
  2.2 Bovine parathyroid cell membrane lysate preparation ..................................51
  2.3 Immunoblotting .......................................................................................51
  2.4 Immunoblot stripping ..............................................................................52
  2.5 DNA transformation into competent cells ................................................53
  2.6 Cell culture ...............................................................................................53
  2.7 Transient cell transfection ........................................................................53
  2.8 Cell assay and lysates preparation ............................................................54
  2.9 Intracellular calcium imaging ....................................................................54
  2.10 Total RNA extraction ..............................................................................55
  2.11 Reverse transcription polymerase chain reaction (RT-PCR) ......................55
  2.12 Immunofluorescence ..............................................................................57
  2.13 Fluorescence resonance energy transfer (FRET) ....................................57
  2.14 Statistical analysis ..................................................................................59

CHAPTER 3 ...........................................................................................................60
Identification of Candidate Components of an Intrinsically-Stimulated
Parathyroid Hormone Secretion Mechanism .....................................................60
  3.1 Introduction ...............................................................................................61
  3.2 Materials and Methods .............................................................................61
  3.3 Results ......................................................................................................61
3.3.1 Expression of GPCRs and related proteins in bovine and murine PT gland as revealed by gene microarray analysis ..................................61
3.3.2 EP4-receptor expression in bovine parathyroid gland .................64
3.3.3 EP4-receptor transfection in CaR-HEK cells .............................66
3.3.4 PGE synthase 1 and 2 expression in bovine parathyroid gland ..........68
3.3.5 PGE2 enhances CaR-induced Ca2+ mobilisation at subthreshold Ca2+o concentration in CaR-HEK cells ..................................69
3.3.6 CALCRL expression in bovine PT gland tissue ..............................71
3.3.7 Other potential cAMP modulating protein expressed in bovine parathyroid gland ........................................................................72
3.3.8 CALCRL transfection in CaR-HEK cells ........................................74
3.3.9 Effect of CGRP addition during CaR-induced Ca2+ mobilisation .........75
3.3.10 Effect of CGRP on CaR-induced Ca2+ mobilisation as a function of extracellular calcium concentration ..................................77
3.3.11 Secondary anti-goat antibody cross-react with bovine parathyroid particulate fractions ..........................................................79
3.3.12 Identification of certain Gαs-coupled GPCRs in murine PT gland preincubated in high, normal or low Ca2+o as revealed by gene microarray analysis ..............................................................80

3.4 Discussion ..........................................................................................83
3.4.1 Identification of candidate intrinsic regulators of PTH secretion ........83
3.4.2 EP4-receptor expression in bovine parathyroid gland .................83
3.4.3 EP4-receptor transfection in CaR-HEK cells .............................84
3.4.4 PGE synthase 1 and 2 expression in bovine parathyroid gland ..........85
3.4.5 PGE2 enhances CaR-induced Ca2+ mobilisation in CaR-HEK cells at subthreshold Ca2+o concentration ..................................86
3.4.6 CALCRL and other potential cAMP-generating receptors in bovine PT gland ..............................................................................87
3.4.7 CALCRL transfection in CaR-HEK cells ........................................87
3.4.8 Effect of CGRP addition during CaR-induced Ca2+ mobilisation .........88
3.4.9 Secondary anti-goat antibody cross-react with bovine parathyroid particulate fractions ..........................................................89

3.5 Summary ..............................................................................................89

CHAPTER 4 ..................................................................................................90

Signal Crosstalk between CaR and Other Parathyroid GPCR Signals ..........90

4.1 Introduction ..........................................................................................91
4.2 Materials and Methods ........................................................................................................91

4.3 Results................................................................................................................................92

4.3.1 Effect of EP<sub>4</sub> receptor co-transfection on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation in CaR-HEK cells ..............................................................92

4.3.2 Effect of Indomethacin on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation in CaR-HEK cells co-transfected with EP<sub>4</sub>R ..............................................94

4.3.3 Effect of EP<sub>4</sub>R co-transfection in CaR-HEK cells on CaR-induced ERK phosphorylation ...........................................................................................................96

4.3.4 Effect of the G<sub>α</sub>s agonist PGE<sub>2</sub> on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation ..................................................................................................................98

4.3.4.1 Effect of PGE<sub>2</sub> on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation as a function of concentration ........................................................................98

4.3.4.2 Effect of PGE<sub>2</sub> on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation as a function of extracellular calcium concentration ......................................................100

4.3.4.3 Effect of PGE<sub>2</sub> on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation at subthreshold Ca<sup>2+</sup><sub>o</sub> concentration .................................................................102

4.3.5 Effect of the G<sub>α</sub>s agonist isoprenaline on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation ............................................................104

4.3.5.1 Effect of isoprenaline on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation as a function of concentration ........................................................................104

4.3.5.2 Effect of isoprenaline on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation as a function of extracellular calcium concentration ..........................................................106

4.3.5.3 Effect of isoprenaline on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation at subthreshold Ca<sup>2+</sup><sub>o</sub> concentration .................................................................108

4.3.6 Effect of the G<sub>α</sub>s agonist histamine on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation ............................................................110

4.3.6.1 Effect of histamine on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation as a function of concentration ........................................................................110

4.3.6.2 Effect of histamine on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation as a function of extracellular calcium concentration ..........................................................112

4.3.6.3 Effect of histamine on CaR-induced Ca<sup>2+</sup><sub>i</sub> mobilisation at subthreshold Ca<sup>2+</sup><sub>o</sub> concentration .................................................................114

4.3.7 PGE<sub>2</sub> treatment enhances protein kinase A activity using anti-PKA phospho-substrate antibody in CaR-HEK cells exposed to moderate Ca<sup>2+</sup><sub>o</sub> concentrations .......................................................................................116

4.3.8 Effect of the PKA inhibitor H89 on PGE<sub>2</sub>-induced PKA activation in CaR-HEK cells treated with low Ca<sup>2+</sup><sub>o</sub> concentration ...........................................118

4.3.9 PGE<sub>2</sub> treatment potentiates extracellular signal-regulated kinase (ERK) phosphorylation and inhibits P70 S6 kinase in CaR-HEK cells exposed to moderate Ca<sup>2+</sup><sub>o</sub> concentrations .......................................................................................120
4.3.10 Selective EP₃R antagonist inhibits PGE₂-induced ERK phosphorylation in CaR-HEK cells ......................................................... 122
4.3.11 The selective β₁ adrenergic receptor antagonist atenolol fails to significantly inhibit isoprenaline-induced ERK phosphorylation in CaR-HEK cells ......................................................... 124
4.3.12 The selective H₁R antagonist pyrilamine inhibits histamine-induced ERK phosphorylation in CaR-HEK cells ......................................................... 126
4.3.13 The additive effect of adding very low concentrations of several Gα₆ agonists on CaR-induced Ca²⁺ᵢ mobilisation in CaR-HEK cells ....... 128
4.4 Discussion ......................................................................................... 130
4.4.1 The hypothesis of intrinsic parathyroid hormone secretion ............ 130
4.4.2 Using CaR-HEK cells as a model to study the crosstalk between CaR and other GPCRs ......................................................... 131
4.4.3 Effect of EP₃R cotransfection on CaR function in CaR-HEK cells .... 133
4.4.4 Parathyroid hormone secretion between Gα₆ and Gα₁₆₁ mediated signalling ......................................................... 134
4.4.5 Effect of cAMP in CaR-mediated Ca²⁺ᵢ mobilisation in subthreshold Ca²⁺₀ concentration ......................................................... 135
4.4.6 Effect of histamine on CaR activation ................................................ 136
4.4.7 Effect of PGE₂ as a cAMP generator in PKA activation in CaR-HEK cells exposed to moderate Ca²⁺₀ concentrations ........................... 137
4.4.8 Biased signalling arising from activation of Gα₆ and Gα₁₆₁-coupled GPCRs ......................................................... 138
4.4.9 The combined stimulatory effect arising from limited stimulation of multiple GPCRs ......................................................... 140
4.5 Summary .......................................................................................... 140
CHAPTER 5 ............................................................................................. 142
CaR-Mediated Modulation of Cyclic AMP Levels in HEK-293 Cells and Human Bronchial Smooth Muscle Cells .......................................................... 142
5.1 Introduction ...................................................................................... 143
5.2 Materials and Methods ..................................................................... 144
5.3 Results ............................................................................................. 144
5.3.1 H187 vector transfection into HEK-293 cells and validation of PGE₂-mediated cAMP accumulation in CaR-HEK cells using FRET ........ 144
5.3.2 Effect of increasing Ca²⁺₀ concentrations on PGE₂-induced cAMP production in CaR-HEK cells transfected with cAMP reporter ....... 146
The CaR positive allosteric modulator R568 enhanced CaR-mediated cAMP inhibition in CaR-HEK cells transfected with cAMP reporter.

Effect of the PKC inhibitor GF102903X on CaR-induced cAMP inhibition.

Effect of PKC inhibition on CaR-induced Ca\(^{2+}\) mobilisation.

Effect of PKC on CaR-induced cAMP suppression in CaR-HEK cells transfected with cAMP reporter.

Effect of the G\(\alpha_q/11\) inhibitor YM-254890 on Ca\(^{2+}\)\(_o\)-induced cAMP suppression in CaR-HEK cells transfected with cAMP reporter.

Effect of the G\(\alpha_i\) inhibitor PTx on high Ca\(^{2+}\)\(_o\)-induced cAMP suppression in CaR-HEK cells transfected with cAMP reporter.

Effect of salbutamol in Ca\(^{2+}\) mobilisation in BSMCs exposed to 2 mM Ca\(^{2+}\)\(_o\) concentration.

Effect of the calcilytic NPS-2143 on salbutamol-induced Ca\(^{2+}\) increase in BSMCs.

Effect of the calcilytic NPS-795 on salbutamol-induced Ca\(^{2+}\) enhancement in BSMCs.

Effect of salbutamol and NPS-2143 on the phosphorylation/activation of ERK, Akt and p38 MAPK in BSMCs exposed to 2 mM Ca\(^{2+}\)\(_o\).

CaR expression in human bronchial smooth muscle cells.

Discussion.

PGE\(_2\) induced cAMP production in CaR-HEK cells as determined by FRET ratiometry.

Effect of CaR activation on PGE\(_2\)-induced cAMP production in CaR-HEK cells transfected with the Epac biosensor.

Role of G\(\alpha_q/11\) mediated signalling in CaR-induced cAMP suppression.

CaR-mediated cAMP suppression is PTx sensitive.

Effect of salbutamol and calcilytics on intracellular calcium mobilisation in BSMCs.

Effect of salbutamol and calcilytics on activation of ERK, Akt and p38 MAPK in BSMCs.

Summary.

CHAPTER 6

General Discussion and Conclusion.

6.1 General discussion.

6.2 General conclusion.
LIST OF FIGURES

Figure 1.1. Active transcellular calcium transport through intestinal epithelial cells ........................................... 24
Figure 1.2. Active calcium reabsorption in the tubular epithelial cells of the distal convoluted tubules in kidney ................................................................. 26
Figure 1.3. PTH induces osteoclast cells differentiation via osteoblast-lineage cells ......................... 30
Figure 1.4. Biased signalling and CaR ................................................................. 40
Figure 1.5. Summary of prostaglandins biosynthesis and intracellular signalling ...................................... 43
Figure 1.6. Summary of histamine biosynthesis and intracellular signalling ............................... 45
Figure 2.1. Model for the conformational change induced by cAMP binding to the regulatory part of Epac detected by FRET ........................................................................ 59
Figure 3.1. EP4 receptor expression in bovine parathyroid gland ..................................................... 65
Figure 3.2. Expression of EP4R in CaR-HEK cells .................................................. 67
Figure 3.3. PGE synthases 1 and 2 expression in bovine parathyroid gland ........................................... 68
Figure 3.4. Effect of PGE2 on CaR-induced Ca2+ i mobilisation at subthreshold Ca2+ o concentration in CaR-HEK cells .................................................. 70
Figure 3.5. CALCRL receptor expression in bovine parathyroid gland ............................................ 72
Figure 3.6. CGRP and adrenomedullin receptor forming components expressed in bovine parathyroid total mRNA................................................................. 73
Figure 3.7. Expression of CALCRL in CaR-HEK cells ..................................................... 74
Figure 3.8. Effect of CGRP in 2.5 mM Ca2+ o-induced Ca2+ i mobilisation ............................... 76
Figure 3.9. Effect of CGRP on Ca2+ o-induced Ca2+ i mobilisation in CaR-HEK cells ......................... 78
Figure 3.10. Secondary anti-goat antibody cross-react with bovine parathyroid particulate fractions ................................................................. 80
Figure 4.1. Effect of increasing Ca2+ o concentration on Ca2+ i mobilisation in CaR-HEK cells co-transfected with EP4R .......................................................... 93
Figure 4.2. Effect of indomethacin on CaR-induced Ca2+ i mobilisation in CaR-HEK cells co-transfected with EP4R ............................................................. 95
Figure 4.3. Effect of increasing Ca2+ o concentrations on ERK phosphorylation in CaR-HEK cells co-transfected with EP4R ............................................................. 97
Figure 4.4. Effect of PGE2 on CaR-induced Ca2+ i mobilisation in CaR-HEK as a function of concentration .............................................................................. 99
Figure 4.5. PGE2 enhances Ca2+ o-induced Ca2+ i mobilisation in CaR-HEK cells ..................... 101
Figure 4.6. Effect of PGE2 and NPS-2143 on subthreshold Ca2+ o induced Ca2+ i mobilisation in CaR-HEK cells ............................................................................ 103
Figure 4.7. Effect of isoprenaline on CaR-induced Ca2+ i mobilisation in CaR-HEK as a function of concentration .............................................................................. 105
Figure 4.8. Isoprenaline enhances Ca2+ o-induced Ca2+ i mobilisation in CaR-HEK cells ......................... 107
Figure 4.9. Effect of isoprenaline and NPS-2143 on subthreshold Ca2+ o induced Ca2+ i mobilisation in CaR-HEK cells ............................................................................ 109
Figure 4.10. Effect of histamine on CaR-induced Ca2+ i mobilisation in CaR-HEK as a function of concentration .............................................................................. 111
Figure 4.11. Effect of histamine on \( \text{Ca}^{2+} \)–induced \( \text{Ca}^{2+} \) mobilisation in CaR-HEK cells. .................................113
Figure 4.12. Effect of histamine and NPS-2143 on subthreshold \( \text{Ca}^{2+} \)–induced \( \text{Ca}^{2+} \) mobilisation in CaR-HEK cells. ........................................115
Figure 4.13. Effect of PGE\(_2\) on PKA substrate phosphorylation in CaR-HEK cells exposed to moderate \( \text{Ca}^{2+} \) concentrations. ....117
Figure 4.14. Effect of the PKA inhibitor H89 on PGE\(_2\)-induced PKA activation in CaR-HEK cells ........................................119
Figure 4.15. PGE\(_2\) treatment potentiates extracellular signal-regulated kinase (ERK) phosphorylation and inhibits P70 S6 kinase in CaR-HEK cells exposed to moderate \( \text{Ca}^{2+} \) concentrations ..........................121
Figure 4.16. Selective EP\(_{4}\)R antagonist inhibits PGE\(_2\)-induced ERK phosphorylation in CaR-HEK cells ........................................123
Figure 4.17. Effect of the selective \( \beta_1\)-adrenergic receptor antagonist atenolol on isoprenaline-induced ERK phosphorylation in CaR-HEK cells ........125
Figure 4.18. Selective H\(_1\)R antagonist inhibits histamine-induced ERK phosphorylation in CaR-HEK cells ........................................127
Figure 4.19. The additive effect of several Go\(_s\) agonists on CaR-induced \( \text{Ca}^{2+} \) mobilisation in CaR-HEK cells ...............................129
Figure 4.20. Schematic diagram proposing how cAMP may elicit self-limiting PTH secretion. .........................................................132
Figure 5.1. Altered CFP/YFP emission ratio in CaR-HEK cells expressing the H187 cAMP sensor in response to PGE\(_2\) ..................................145
Figure 5.2. Increasing \( \text{Ca}^{2+} \) concentrations reverse PGE\(_2\)-induced cAMP production in CaR-HEK cells. ........................................147
Figure 5.3. The CaR positive allosteric modulator R568 enhanced CaR-mediated cAMP inhibition in CaR-HEK cells .............................149
Figure 5.4. PKC inhibition by GF102903X enhances CaR-induced \( \text{Ca}^{2+} \) mobilisation in CaR-HEK .................................................152
Figure 5.5. PKC inhibition with GF102903X did not enhance the CaR-mediated cAMP suppression in CaR-HEK cells transfected with Epac vector....153
Figure 5.6. Effect of the Go\(_{q/11}\) inhibitor YM-254890 on high \( \text{Ca}^{2+} \)–induced cAMP suppression in CaR-HEK cells transfected with Epac vector ..........155
Figure 5.7. Effect of the Go\(_i\) inhibitor PTx on high \( \text{Ca}^{2+} \)–induced cAMP suppression in CaR-HEK cells transfected with Epac vector ..........157
Figure 5.8. Salbutamol enhances \( \text{Ca}^{2+} \)–induced \( \text{Ca}^{2+} \) enhancement in human BSM cells. .........................................................159
Figure 5.9. Salbutamol failed to significantly enhance \( \text{Ca}^{2+} \) mobilisation in low \( \text{Ca}^{2+} \) concentration in BSMCs ........................................160
Figure 5.10. Effect of the calcilytic NPS-2143 on salbutamol-enhanced \( \text{Ca}^{2+} \) concentrations in BSMCs .............................................162
Figure 5.11. Effect of the calcilytic NPS-795 on salbutamol-enhanced \( \text{Ca}^{2+} \) concentrations in BSMCs .............................................164
Figure 5.12. Effect of salbutamol and NPS-2143 on the phosphorylation levels of ERK, Akt and p38MAPK in BSMCs exposed to 2 mM Ca^{2+}. ............... 166
Figure 5.13. CaR expression in human BSMCs. ............................................. 168
Figure 5.14. Schematic diagram of intracellular signalling involved upon the activation of CaR and β-adrenergic receptor in BSMCs. ....................... 174
Figure 6.1. Hypothetical model of how endogenous prostanoids may elicit autocrine / paracrine stimulation of EP_4 and prostacyclin receptors. .............. 177
LIST OF TABLES

Table 1.1. Diseases resulting from mutation of the calcium-sensing receptor .......... 37
Table 2.1. Primers sequences and RT-PCR products sizes .................................. 56
Table 3.1. Validation of bovine and murine gene microarrays................................. 62
Table 3.2. Highly probable expressed genes in bovine and murine microarray analysis .......................................................... 63
Table 3.3. Table of selected receptors and their ligand synthases from mouse microarray data .......................................................... 82
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_2$AR</td>
<td>Beta 2 adrenergic receptor</td>
</tr>
<tr>
<td>$1,25$(OH)$_2$D$_3$</td>
<td>1,25-dihydroxyvitamin D$_3$</td>
</tr>
<tr>
<td>$2^\circ$HPT</td>
<td>Secondary hyperparathyroidism</td>
</tr>
<tr>
<td>20-HETE</td>
<td>20-Hydroxyeicosatetraenoic acids</td>
</tr>
<tr>
<td>25(OH)$_2$D$_3$</td>
<td>25-hydroxyvitamin D$_3$</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADH</td>
<td>Autosomal dominant hypoparathyroidism</td>
</tr>
<tr>
<td>AM</td>
<td>Adrenomedullin</td>
</tr>
<tr>
<td>AMV</td>
<td>Avian myeloblastosis virus reverse transcriptase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMU</td>
<td>Basic multicellular unit</td>
</tr>
<tr>
<td>bPTG</td>
<td>Bovine parathyroid gland</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSMCs</td>
<td>Bronchial smooth muscle cells</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>Ca$_{\text{i}}^{2+}$</td>
<td>Intracellular calcium</td>
</tr>
<tr>
<td>Ca$_{\text{o}}^{2+}$</td>
<td>Extracellular calcium</td>
</tr>
<tr>
<td>CALCRL</td>
<td>Calcitonin receptor-like receptor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CaR</td>
<td>Calcium sensing-receptor</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan fluorescent protein</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>cm</td>
<td>Calcimimetic</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>Cx</td>
<td>Calcilytic</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DCT</td>
<td>Distal convoluted tubules</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECP</td>
<td>Eosinophilic cationic protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
</tr>
<tr>
<td>EP₄R</td>
<td>Prostanoid receptor subtype 4</td>
</tr>
<tr>
<td>Epac</td>
<td>Exchange factor directly activated by cAMP</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESRD</td>
<td>End stage renal disease</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FHH</td>
<td>Familial hypocalciuric hypercalcemia</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>GFX</td>
<td>GF102903X</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HEK-293 cells</td>
<td>Human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular domain</td>
</tr>
<tr>
<td>IGFR</td>
<td>Insulin-like growth factor receptor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>IP₃R</td>
<td>Inositol trisphosphate receptor</td>
</tr>
<tr>
<td>IPR</td>
<td>Prostacyclin receptor</td>
</tr>
<tr>
<td>iPTH</td>
<td>Intact parathyroid hormone</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>L-Phe</td>
<td>L-Phenylalanine</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCT</td>
<td>Medullary collecting tubules</td>
</tr>
<tr>
<td>MEN-1</td>
<td>Multiple endocrine neoplasia-1</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium calcium exchanger</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NSHPT</td>
<td>Neonatal severe primary hyperparathyroidism</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PGG&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin endoperoxide</td>
</tr>
<tr>
<td>PGH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin H&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PGs</td>
<td>Prostaglandins</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositide 3-kinases</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PMA</td>
<td>Present/Margin/Absent</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane calcium ATPase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethane sulfonyl fluoride</td>
</tr>
<tr>
<td>PNGase</td>
<td>Peptide-N-Glycosidase</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PT</td>
<td>Parathyroid</td>
</tr>
<tr>
<td>PTG</td>
<td>Parathyroid gland</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PTH-1 R</td>
<td>Parathyroid hormone-1 receptor</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone related protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PTx</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>RAMP</td>
<td>Receptor activity-modifying protein</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor κB</td>
</tr>
<tr>
<td>RANK-L</td>
<td>Receptor activator of nuclear factor κB ligand</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay buffer</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TRPC1</td>
<td>Transient receptor potential channel 1</td>
</tr>
<tr>
<td>TRPV</td>
<td>Transient receptor potential vanilloid</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
</tr>
<tr>
<td>TXs</td>
<td>Thromboxanes</td>
</tr>
<tr>
<td>VFT</td>
<td>Venus fly trap</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
University: University of Manchester
Name: Halah Tariq Albar
Degree title: Doctor of Philosophy (PhD)
Thesis title: Signal Crosstalk between Calcium-Sensing and Prostanoid Receptors and its Role in Parathyroid Hormone Secretion
Date: December 2017

ABSTRACT
The calcium-sensing receptor (CaR) maintains extracellular calcium Ca$^{2+}$ homeostasis by suppressing parathyroid hormone (PTH) secretion. However, the underlying cellular driving force for tonic PTH secretion remains unclear. It is believed that agonists that generate intracellular cyclic-AMP levels may increase PTH secretion and therefore bidirectional crosstalk between CaR signalling and cAMP levels could be of physiological importance.

In order to identify possible contributors to parathyroid cAMP accumulation I first confirmed the gene and protein expression of EP$_4$R (prostanoid) and CALCRL (calcitonin receptor-like receptor) in bovine PT gland by using RT-PCR and western blotting respectively. Also, I confirmed the gene expression of the PGE synthase enzyme required for the PGE$_2$ synthesis in bovine PT gland by RT-PCR. And I further confirmed the gene expression of the CALCRL’s associated components particularly the RAMPs and adrenomedullin.

Next, I investigated the influence of cAMP on CaR signalling as determined first by Ca$^{2+}$ mobilisation. Cyclic AMP-generating agonists including PGE$_2$, isoprenaline and histamine enhanced CaR-induced Ca$^{2+}$ mobilisation at subthreshold Ca$^{2+}$o concentrations. Indeed, PGE$_2$ and isoprenaline lowered the EC$_{50}$ for Ca$^{2+}$o-induced Ca$^{2+}$i mobilisation (2.5 ± 0.1 for PGE$_2$ and 2.7 ± 0.2 for isoprenaline vs. 4.0 ± 0.3 mM for control, P<0.01) in CaR-HEK cells using Fura2-based microfluorimetry. In addition, PGE$_2$ significantly enhanced the CaR-mediated phosphorylation of extracellular signal-regulated kinase (ERK) but tended to inhibit P70 S6 kinase in CaR-HEK cells exposed to moderate Ca$^{2+}$o concentrations. PGE$_2$ specifically enhanced ERK1/2 phosphorylation via activation of EP$_4$R as this effect was inhibited with the selective EP$_4$R antagonist L161982 (p<0.01). Then, cotreatment with three Ga$_s$-agonists at subthreshold concentrations significantly enhanced CaR-induced Ca$^{2+}$i mobilisation in the presence of 2 mM Ca$^{2+}$o whereas each was without effect on its own. This raises the possibility that CaR signalling could be modulated by modest physiological stimuli acting through a variety of Ga$_s$-coupled GPCRs. Then using a FRET-based cAMP reporter (based on Epac) it was shown that PGE$_2$ stimulates cAMP accumulation in CaR-HEK cells while high Ca$^{2+}$o- or R568-mediated CaR activation lowers cAMP levels via a pertussis toxin-sensitive signalling pathway. Furthermore, the CaR-induced decrease in intracellular cAMP levels was not altered by PKC inhibition, despite this intervention increasing CaR-induced Ca$^{2+}$i mobilisation.

Finally, there is evidence that CaR-inhibiting calcilytic drugs might treat asthma by antagonising the effects of cationic compounds on CaR activation in airway hyper responsiveness disease. Therefore, here I first showed that increasing Ca$^{2+}$o concentration in human bronchial smooth muscle cells (BSMCs) permits the β2AR agonist salbutamol to increase Ca$^{2+}$i mobilisation and inhibit ERK1/2 phosphorylation. However, neither effect could be inhibited by calcilytic cotreatment suggesting that the Ca$^{2+}$o effect does not occur via CaR activation in these BSMCs derived from a non-asthmatic donor.

The major finding of the present study is that there is significant crosstalk between CaR and Ga$_s$-coupled GPCR signalling pathways with modest concentrations of cAMP-generating agonists potentiating CaR-induced Ca$^{2+}$i mobilisation and CaR stimulation attenuating enhanced cAMP levels. This may be of greatest relevance in vivo where endogenous agonists of Ga$_s$-coupled GPCRs may be more abundant than in the simple salt solutions used in vitro. Together, this work provides novel insights into the possible driving force for PTH secretion as well as the CaR-mediated regulation of secretion.
DECLARATION

No portion of the work in this doctoral thesis has been submitted in support of an application for another degree or qualification at the University of Manchester or any other university or other institute of learning.
COPYRIGHT STATEMENT

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and she has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DocuInfo.aspx?DocID=24420), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.library.manchester.ac.uk/about/regulations/) and in The University’s policy on Presentation of Theses.
In the name of Allah, the Most Gracious and the Most Merciful

Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis. Special appreciation goes to my supervisor Dr. Donald Ward for his outstanding supervision. He has been always patient, understanding, helpful, and cooperative throughout the writing and research process. Without his endless support I would not have completed my programme requirements smoothly.

Special thanks go to Jeffrey Klarenbeek (Amsterdam) for providing the cAMP FRET reporter.

The Bioimaging Facility microscopes used in this study were purchased with grants from BBSRC, Wellcome and the University of Manchester Strategic Fund. Special thanks goes to [Peter March / Roger Meadows/ Steven Marsden] for their help with the microscopy.

I would like to thank my sponsor: Umm Al-Qura University, Makkah, Saudi Arabia.

I would like to express my endless thanks to my mother Salwa and my Father Tariq for everything they have done to get me to where I am at today. A special thanks go to my sisters; Heba, Hadiel, and Shahad who have always motivated me to strive towards my goals.

Last but not least, I want to extend my thanks to everyone who has helped and supported me throughout all my endeavours especially my friends Boudor Rajab and Jawaher Alshehri.

Thank you
CHAPTER 1

Introduction
1.1 Introduction

Maintaining calcium homeostasis in both extracellular and intracellular compartments is crucial in the human body. Calcium homeostasis is essential for most of the physiological functions in the body (Brown, 2013). For example, calcium is the major constituent of the bone and it is also involved in nerve conduction, muscle contraction, intracellular signalling, enzymes and other actions such as in blood clotting (Brown, 2013; Blaine et al., 2015; Beggs and Alexander, 2017). Alterations in calcium homeostasis can lead to serious pathological conditions from arrhythmias and tetany to osteoporosis and nephrolithiasis (Haghighi et al., 2013; Han et al., 2015). The form of calcium that the body directly attempts to control homeostatically is the free ionised calcium present in the plasma, which is controlled within a narrow range between 1.1 to 1.2 mM (Conigrave, 2016). The fundamental mediator of calcium homeostasis is parathyroid hormone (PTH) which increases blood Ca\(^{2+}\) concentrations by acting on three main organs; bone, kidney and intestine (Brown, 2013; Conigrave, 2016). But it is the negative feedback regulator of PTH secretion, a G protein-coupled receptor called the extracellular calcium-sensing receptor (CaR), which represents the key controller of extracellular calcium homeostasis (Ward and Riccardi, 2012; Mantovani et al., 2017). Extracellular calcium (Ca\(^{2+}\))-dependent CaR activation results in activation of the Ga\(_{q/11}\)/phospholipase C (PLC) intracellular signalling pathway which in turn leads to the generation of inositol trisphosphate (IP\(_3\)) and diacylglycerol (DAG). Then, IP\(_3\) binds to IP\(_3\)R present on the surface of endoplasmic reticulum enhances Ca\(^{2+}\) mobilisation whereas DAG formation causes PKC activation (Nemeth and Scarpa, 1987; Ward, 2004; Hannan et al., 2017). This and other CaR-induced signals such as the activation of mitogen-activated protein kinases (MAPK) pathway are involved in PTH secretion inhibition (Ward, 2004; Conigrave, 2016). In contrast, the essential cellular driving force responsible of tonic PTH secretion in the first place remains largely unknown.

1.2 Physiology of Calcium homeostasis

In the event of hypocalcaemia, PTH increases blood Ca\(^{2+}\) concentration by increasing the activity of osteoclast cells in the bones and, by increasing calcium
reabsorption in the kidney tubules (Carmeliet et al., 2003; Blaine et al., 2015). PTH also indirectly increases calcium absorption from the intestine by increasing 1α-hydroxylase activity in the renal proximal tubule and thus producing more of the active form of vitamin D₃, namely 1,25(OH)₂D₃ (Carmeliet et al., 2003; Diepenhorst et al., 2017). In contrast, under conditions of hypercalcaemia, the suppression of PTH secretion results in attenuated bone catabolism, decreased renal calcium reabsorption and less 1-α hydroxylation of 25(OH) vitamin D₃ (Blaine et al., 2015).

1.3 Calcium distribution between kidney, bone, intestine and extracellular fluid (ECF)

The human body holds about 1-1.3 kg of calcium, around 99% of calcium being present in the bones and teeth, mostly in the form of hydroxyapatite \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\) (Zhu and Prince, 2012; Song, 2017), with the remaining 1% being located in the plasma and intracellular fluid. Less than 50% of calcium present in the plasma is non-diffusible, being bound to different proteins such as albumin and globulin (Baird, 2011; Blaine et al., 2015; Song, 2017). Whereas, the other 50% is either free and ionised, or exists complexed with bicarbonate, citrate or lactate organic anions (Baird, 2011; Agrò, 2013). It is this ionised component of serum calcium that is tightly controlled within a small range, generally 1.1-1.3 mM (Conigrave et al., 2008; O’Toole, 2011) representing its optimal physiological level. The plasma protein albumin also acts as a buffer to keep the level of the ionised calcium within normal range. Thus, if there is a drop in the ionised calcium concentration, more calcium can be released from its binding sites on the albumin to ameliorate the drop in the ionised calcium concentration (Baird, 2011).

1.3.1 Intestinal calcium absorption

Calcium absorption in the intestine occurs mainly in the duodenum followed by the jejunum and is affected by many factors such as serum calcium and vitamin D₃ concentration (O’Toole, 2011), as well as puberty, pregnancy and lactation (Zhu and Prince, 2012). There are two routes for calcium absorption in the intestine, passive paracellular transport and active transcellular pathway (Bronner, 2003; Blaine et al., 2015). Passive paracellular calcium absorption is dependent on the level of calcium
inside the intestinal lumen, that is, the dietary calcium content (Bronner, 2003).

However, the active transcellular transport occurs uphill against the concentration gradient of calcium requiring ATP as a source of energy (Bronner, 2003; Beggs and Alexander, 2017), and depends on the cell’s prior exposure to 1,25(OH)₂ vitamin D₃ (Sahota and Hosking, 1999). This transcellular active transport begins with apical calcium entry mediated chiefly by the membrane calcium channel TRPV6 located in the apical brush border of both duodenum and jejunum epithelial cells. Next, the calcium binds to the carrier protein Calbindin-D₉K inside the cell until it is released from the cell through the basolateral membrane by the plasma membrane calcium ATPase PMCA and via the sodium calcium exchanger, NCX (Sahota and Hosking, 1999; Fleet, 2017) (Figure 1.1). The active form of vitamin D₃ stimulates the synthesis of several of these proteins including TRPV6 and calbindin-D₉K (Blaine et al., 2015; Christakos et al., 2016) (Figure 1.1).

**Figure 1.1. Active transcellular calcium transport through intestinal epithelial cells**

Active transcellular transport of Ca²⁺ occurs mainly in low calcium diet. First Ca²⁺ enters the cell through TRPV6 channels located in the apical bush border of the intestinal epithelial cells. Then, Ca²⁺ binds to the carrier Calbindin-D₉K inside the cell until it is released into the blood across the basolateral membrane via PMCA and NCX. 1,25(OH)₂ vitamin D₃ increases the expression level of TRPV6 and calbindin-D₉K.
1.3.2 Renal calcium reabsorption

Almost 99% of filtered calcium is reabsorbed in renal tubules (Bronner, 2003; Khalil et al., 2017). Around 60-70% of calcium filtered load is reabsorbed passively through paracellular transport in the proximal tubule of the kidney (Jeon, 2008; Christakos et al., 2016). This occurs due to the solvent drag properties of sodium and water movement and because the calcium concentration is relatively high in the tubular lumen (O’Toole, 2011; Blaine et al., 2015). The calcium reabsorption in the distal convoluted tubules and collecting duct is influenced by relative parathyroid hormone, calcitonin, and vitamin D₃ concentrations (Sahota and Hosking, 1999). About 10% of calcium filtered load is reabsorbed in the distal convoluted tubules (DCT) via the active transcellular transport pathway (O’Toole, 2011; Blaine et al., 2015; Khalil et al., 2017). The calcium active transport in the kidney is quite similar to that in the intestine, except that the apical entry channel is TRPV5 and the cytosolic carrier protein Calbindin-D₂₈K; the final step occurring via PMCA as before (Bronner, 2003; Carmeliet et al., 2003; O’Toole, 2011; Khalil et al., 2017) (Figure 1.2).

In addition, the last step of the activation of vitamin D₃ (1-α hydroxylation) also takes place in proximal tubules of the kidney, this then acting to stimulate intestinal calcium absorption (Friedman, 1999; O’Toole, 2011; Berridge, 2015).
First $\text{Ca}^{2+}$ enters the cell through TRPV5 channels situated in the apical membrane of the DCT epithelial cells. Then, $\text{Ca}^{2+}$ binds to the carrier Calbindin-D$_{28K}$ in the cytoplasm until it is released into the blood across the basolateral membrane via PMCA and NCX.

### 1.3.3 Skeletal mineral storage

Bone plays a very important role in calcium homeostasis and to achieve this role the bone is constantly undergoing remodeling (Kular et al., 2012). The remodeling process happens in a basic multicellular unit (BMU) consisting of two types of cells, i) the osteoclasts, which are bone resorbing cells and ii) the osteoblasts, which are the bone forming cells (Song, 2017). These two cells are working under the influence of many hormones such as; PTH, calcitonin and the active form of vitamin D$_3$. Indeed, the movement of mobilisable calcium in and out of the bone plays a major role in controlling the minute-to-minute adjustment of serum calcium levels (Bronner, 2003; Song, 2017).

During the bone resorbing process, the osteoclast’s lamellipodia attach to a specific portion of bone, where they release acid ($\text{H}^+$) and lysosomes, causing the bone salts to become more soluble thus helping to destroy the bone matrix (Kular et al., 2012; Meleleo and Picciarelli, 2016). Then, the calcium concentration around the cell becomes high, stopping the function of the lamellipodia and causing the cell to migrate to another location and restart its function (Väänänen and Zhao, 2002; Bronner, 2003). When the osteoclast’s calcitonin receptor is activated in response to calcitonin, the osteoclast cells contract losing their activity and thus the plasma
calcium concentrations fall (Bronner, 2003). In contrast the osteoblasts, which are the only bone cells expressing parathyroid hormone receptors will, in response to PTH, contract exposing the low affinity calcium binding sites and thus the plasma calcium concentration will rise (Bronner, 2003). The osteoblast cells also have vitamin D receptors which respond to the active form of vitamin D$_3$ in a way parallel to their response to PTH although the response to vitamin D$_3$ is much slower (Bronner, 2003; Nakamichi et al., 2017).

### 1.4 Endocrine control of serum calcium concentration

#### 1.4.1 Parathyroid hormone

Serum calcium concentration is tightly controlled by the action of three hormones; PTH, calcitonin and 1,25(OH)$_2$ vitamin D$_3$ (Cline, 2012; Blaine et al., 2015). However, it is the PTH that represents the main regulator of serum calcium levels in the body (Gensure et al., 2005). PTH is produced from chief cells of the parathyroid gland which is located behind the thyroid gland in the neck (Campbell, 2011). PTH is a polypeptide chain comprising 84-amino acids in its mature form (Summers and Macnab, 2017). The calcium-sensing receptor (CaR) present on the surface of the Chief cells of the parathyroid gland is highly sensitive to the serum ionised calcium concentration. When the blood calcium concentrations declines e.g. to 1 mM, PTH secretion by the gland is markedly increased (Campbell, 2011; Conigrave, 2016). The PTH then acts to increase blood calcium levels again by eliciting its action on bone and kidney (Kim et al., 2014; Blaine et al., 2015).

#### 1.4.2 Vitamin D

Vitamin D$_3$ is a steroid-like hormone (Bronner, 2003; Blaine et al., 2015), formed in the body as a result of the cooperation of three organs; skin, liver, and kidney (Henry, 2011; Berridge, 2015). The skin epidermis contains 7-dehydrocholesterol which turns into vitamin D$_3$ (cholecalciferol) after exposure to the sun’s ultraviolet light (Henry, 2011; Blaine et al., 2015). Then, in the liver, vitamin D$_3$ undergoes hydroxylation in the C-25 position resulting in the formation of 25(OH)D$_3$ the most abundant form of vitamin D$_3$ in the circulation (Henry, 2011; Christakos et al., 2017). Finally, in the kidney, further hydroxylation of 25(OH)D$_3$ occurs via the
enzyme 1α-hydroxylase in the proximal convoluted tubules resulting in the formation of the active form of vitamin D₃ 1,25(OH)₂D₃, also known as calcitriol (Christakos et al., 2017).

When calcium requirements are increased during growth, pregnancy, lactation, or when consuming a calcium-deficient diet, the production of active vitamin D₃ by the kidney is increased (Cooper, 2011). Active vitamin D₃ then increases serum calcium concentrations mainly by increasing calcium intestinal absorption. Accordingly, in the case of vitamin D₃ deficiency, intestinal calcium absorption is decreased leading to osteoporosis and in more severe cases to a delay in cartilage and osteoid calcification leading to rickets in children or osteomalacia in adults (Nordin, 1990; Christakos et al., 2017).

1.4.3 Calcitonin

Calcitonin is a polypeptide hormone containing 32 amino acids secreted from the parafollicular C cells of the thyroid gland in response to hypercalcemia (Campbell, 2011; Summers and Macnab, 2017). Calcitonin lowers the serum calcium concentration by opposing the effect of PTH and vitamin D₃ (Baird, 2011). In bones, calcitonin mediates its action through calcitonin receptors present on the cell membrane of osteoclast cells (Bronner, 2003). Calcitonin inhibits the action of osteoclasts by decreasing its binding affinity with bone surfaces resulting in loss of the ruffled border and decreased activity of the acid-bearing lysosomes (Sahota and Hosking, 1999; Kitay and Geibel, 2017). This effect favors the action of bone-forming cells, the osteoblasts that lead to bone mineralization (Summers and Macnab, 2017). In kidney, calcitonin increases the urine excretion of calcium in distal tubules and down-regulates the activation of vitamin D₃ in proximal tubules. While being essential in salt-water fish faced with the challenge of life in a 10 mM Ca²⁺-containing environment, in terrestrial animals calcitonin is of less importance since the challenge on dry-land is to keep blood calcium concentration sufficiently high (Hirsch and Baruch, 2003; Guh and Hwang, 2016). Indeed there are no known (non-malignant) endocrine pathologies relating to high or low calcitonin levels (Bronner, 2003).
1.5 Parathyroid hormone

1.5.1 Parathyroid hormone function

Parathyroid hormone (PTH), is the main regulator of extracellular calcium concentration (Conigrave, 2016). The parathyroid glands are highly sensitive to changes in plasma calcium concentrations and when serum calcium drops below normal levels, the parathyroid gland secretes PTH into the blood (Gardella and Kronenberg, 2004; Conigrave, 2016). Then, the PTH raises the calcium concentration back to normal by binding to the PTH-1 receptors expressed on the surface of osteoblasts and certain renal target cells (Milstrey et al., 2017).

Following binding to the G protein-coupled receptor PTH-1 receptor, PTH stimulates the enzyme adenyl cyclase (AC), via Ga₃s, to increase cyclic AMP levels inside the cell resulting in activation of protein kinase A (Alexander et al., 2017). However, at higher PTH concentrations, PTH1-R can also couple to the phospholipase C (PLC)/inositol trisphosphate (IP₃)/intracellular Ca²⁺/PKC intracellular pathway through the activation of Gaq/11 (Kitay and Geibel, 2017).

PTH can elicit two different actions on the bone, either anabolic or catabolic effect depending on the dose and timing of secretion (Milstrey et al., 2017). PTH induces bone resorption indirectly via osteoblasts, which are the only PTH1-R-expressing bone cells, when the osteoblasts receptor activator of nuclear factor κB ligand, RANK-L binds to its receptor RANK on osteoclasts (Figure 1.3). Then, osteoclasts precursor cells differentiate to mature osteoclasts which in turn causes bone resorption (Fu et al., 2017). However, another factor named Osteoprotegerin (OPG) can inhibit this effect and the ratio between the OPG and RANKL is defined by the manner of PTH administration (Qin et al., 2004; Kitay and Geibel, 2017; Nakamichi et al., 2017). In the case of continuous (generally pathological) PTH release, the RANKL:OPG ratio increases up to 25-fold with a net increase in bone demineralisation (Qin et al., 2004). In contrast, when PTH is secreted intermittently it will induce osteoblast cells differentiation, rather than osteoclasts differentiation, with a net increase in bone formation and mineralisation and in this case the PTH has only a small effect on the RANKL:OPG ratio (Qin et al., 2004).
Figure 1.3. PTH induces osteoclast cells differentiation via osteoblast-lineage cells

PTH acts on osteoblasts via PTH-1 R to induce expression of RANK-L. RANK-L binds to RANK (receptor for RANK-L) expressed on osteoclast precursors and thus induces osteoclasts differentiation. Osteoblasts secrete OPG (decoy receptor for RANK-L) which inhibits RANK-RANK-L interaction.

In kidney, PTH increases both the reabsorption of calcium and the excretion of phosphate (Gardella and Kronenberg, 2004). In distal tubule, PTH increases the calcium reabsorption via increasing the activity of calcium ATPase pump PMCA (Blaine et al., 2015). Also, in the proximal tubules PTH increases phosphate excretion (to decrease the risk of calcium phosphate precipitation occurring in the blood) and the formation of active vitamin D₃ which stimulates more calcium intestinal absorption (Gardella and Kronenberg, 2004).

1.5.2 Regulation of parathyroid hormone secretion

Parathyroid gland responds to hypocalcaemia according to the duration, magnitude and the rapidity of the hypocalcaemic stress (Brown, 2000). PTG initially responds within seconds to the acute attack of hypocalcaemia by releasing stored PTH from the secretory vesicles (Brown, 2000; Conigrave, 2016). This response lasts for about half an hour until depletion of stored PTH occurs. After 20-30 minutes from the onset of hypocalcaemic stress, PTG starts to decrease the rate of PTH degradation resulting in more secretion of mature PTH (1-84) (Brown, 2000). PTH breakdown
increases the production of the biologically inactive NH$_2$-truncated PTH peptide fragments (Friedman and Goodman, 2006). Persistent hypocalcaemia that lasts for several hours to days enhances stability of the mRNA encoding the preproPTH (Brown, 2000). Subsequently, the biosynthetic capacity of PT cells is increased and the cells undergo some modification such as enlarged ER and Golgi apparatus. If hypocalcaemia is maintained for longer (several days to weeks), PTG increases the cell’s proliferation capacity particularly chief cells and accordingly produces more PTH (Brown, 2000).

On the other hand, hypercalcaemia activates CaR resulting in activation of several G-proteins including Ga$_{q/11}$, Ga$_i$ and Ga$_{12/13}$ (Ward, 2004; Diepenhorst et al., 2017). CaR-mediated activation of Ga$_{q/11}$ stimulates PLC activation and thus PKC activation. Then, the activated PKC induces the activation of the MAPK cascade which includes several serine/threonine-specific protein kinases such as ERK1/2, cJun NH$_2$-terminal kinases and p38$^{\text{MAPK}}$ (Drüeke, 2004). Afterwards, ERK1/2 activates the cytoplasmic phospholipase A$_2$ in the cell membrane to produce arachidonic acid (AA) which are subsequently metabolised into hydroxyperoxyeicosatetraenoic and hydroxyeicosatetraenoic acids (20-HETE). These two metabolites modulate PTH secretion inhibition (Bourdeau et al., 1992; Bourdeau et al., 1994).

1.5.3 Diseases of parathyroid hormone secretory dysfunction: primary and secondary hyperparathyroidism

Parathyroid gland diseases occur when the gland’s activity is altered resulting in more or less PTH secretion. Hyperparathyroidism is associated with hypersecretion of parathyroid hormone (Bilezikian et al., 2016; Yeung and Tahir, 2017) and it is termed primary when it arises in the absence of any external input or systemic pathology resulting in an apparent hypocalcemia such as chronic kidney disease, liver or intestinal disease (LiVolsi and Baloch, 2004; Fraser, 2009; Yeung and Tahir, 2017).

Single parathyroid adenoma is the most common cause of primary hyperparathyroidism and it represents around 85% of clinical cases (Åkerström and Hellman, 2004; Yeung and Tahir, 2017). There are a range of possible causes of
primary hyperparathyroidism including neck radiation and mutations in multiple endocrine neoplasia-1 (MEN1) (Fraser, 2009; Bilezikian et al., 2016). The second type of primary hyperparathyroidism involves parathyroid hyperplasia which represents around 10-15% of all cases (LiVolsi and Baloch, 2004). In parathyroid hyperplasia, the parenchymal cells of the gland proliferate without the presence of any external stimulus. Parathyroid carcinoma represents 1-2% (Åkerström and Hellman, 2004; Goswamy et al., 2016; Campenni et al., 2017; Gao et al., 2017) of primary hyperparathyroidism cases and it affects both sexes equally (Campenni et al., 2017). Patients usually present with a neck mass associated with systemic symptoms related to hypercalcemia such as kidney stones, osteoporosis and renal failure (LiVolsi and Baloch, 2004; Campenni et al., 2017; Sun et al., 2017). Parathyroid carcinoma is composed of chief cells in most cases however, some oxyphil cells and transitional cells may be present (LiVolsi and Baloch, 2004; DeLellis, 2005).

Patients with primary hyperparathyroidism are usually asymptomatic but they can present with kidney stones that develop due to chronic hypercalciuria arising from the sustained hypersecretion of PTH (Fraser, 2009). Other symptoms of chronic hypercalcemia such as osteoporosis, acute pancreatitis (Gao et al., 2017), depression and dementia are less common consequences of primary hyperparathyroidism (Fraser, 2009; Gao et al., 2017). The treatment of choice for primary hyperparathyroidism is parathyroid (minimally-invasive) surgical excision and these are generally curative (Fraser, 2009; Bilezikian et al., 2016). However, in patients who are not eligible for surgery, medical treatment should be considered including different drugs such as; i) bisphosphonates to increase the density of trabecular bone ii) calcimimetics which bind to CaR in the parathyroid gland and thus return serum calcium level to normal (Peacock et al., 2011; Saponaro et al., 2013) iii) vitamin D analogues (alfacalcidol) to increase vitamin D receptor expression and thus reduce the PTH levels without raising the serum calcium levels, are also considered (Åkerström and Hellman, 2004; Khan et al., 2004; Khan et al., 2009; Bilezikian et al., 2016).

As mentioned earlier, secondary hyperparathyroidism (2°HPT) maybe associated with abnormal function of any organ involved in calcium homeostasis (Yeung and
Patients with 2°HPT develop symptoms related to hypersecretion of PTH and persistent hypocalcaemia. In the case of chronic kidney disease (CKD), 2°HPT occurs as an adaptation to the deterioration in renal function (Friedl and Zitt, 2017). In stage 2 of chronic kidney disease, when the glomerular filtration rate (GFR) is between 60-89 ml/min/1.73m$^2$, serum 1,25(OH)$_2$D$_3$ levels begin to gradually decline (Romagnani et al., 2017). With more progression of the disease, the activity of the enzyme 1α-hydroxylase is further diminished due to hyperphosphataemia, hyperuricaemia, metabolic acidosis and 25(OH)D$_3$ deficiency (Melamed et al., 2016). Hypocalcaemia starts to develop when GFR is <50 ml/min/1.73m$^2$ and causes further increases in PTH secretion. The major symptoms associated with CKD-induced 2°HPT are bone resorption, fractures and cardiovascular diseases (Fraser, 2009). The goals of the treatment in CKD are to; i) slow the progression of the disease and ii) ameliorate the disease complications (Fraser, 2009). In CKD, dialysis is the first line of management to control the serum phosphate concentrations (Blaine et al., 2015). Administration of vitamin D analogues decrease PTH levels and improve the bone mineral density in patients with CKD (Webster et al., 2017). Also, the calcimimetic Cinacalcet has been proven to reduce fracture incidence as well as the need for parathyroidectomy and hospitalisation in CKD patients (Fraser, 2009; Portillo and Rodríguez-Ortiz, 2017; Romagnani et al., 2017).

### 1.6 Calcium-sensing receptor

#### 1.6.1 Structure

The human CaR consists of 1078 amino acids (Goodman, 2004; Hannan et al., 2016) and its amino acid sequence is highly similar to that in bovine CaR (Garrett et al., 1995). Human CaR is known to have 11 N-glycosylation sites in its extracellular domain (ECD) whereas in bovine CaR only 9 of the N-glycosylation sites are conserved (Ray et al., 1998). Organs involved in calcium homeostasis tend to express CaR endogenously at high levels, although CaR is also expressed in many other tissues such as placenta, brain, skin, pancreas and pituitary albeit at lower abundance (Mamillapalli and Wysolmerski, 2010; Riccardi and Kemp, 2012; Alfadda et al., 2014). CaR is a member of the family C of GPCRs and these have
distinctively very large extracellular N-terminal domains (Brown et al., 1993). The CaR consists of 4 major parts; the extracellular N-terminal Venus Fly Trap (VFT) domain, cysteine-rich domain (both in the extracellular domain), heptahelical domain and the intracellular carboxy-terminal domain (Conigrave and Ward, 2013).

The extracellular domain (ECD) consists of around 600 amino acids (Chang and Shoback, 2004) and it contains ligand-binding sites which play an important role in the sensing of Ca\(^{2+}\) (Brown, 2007). The extracellular domain has several N-glycosylation sites which are important for CaR expression on the cell surface (Tfelt-Hansen and Brown, 2005; Ray, 2015). The VFT domain consists of two lobe-shaped domains LB1 and LB2 (Geng et al., 2016). The calcium-binding sites are present in the cleft between the 2 globular lobes forming the venus fly trap domain. Ca\(^{2+}\) binding closes the cleft between the 2 lobes and changes the conformation of the cysteine-rich region which is connected to the transmembrane heptahelical domain and thus activates the G protein-dependent signalling (Hendy et al., 2009). However, recently in 2016, Geng et al. reported that the integration between aromatic amino acids, PO\(_4\)\(^{3-}\) and Ca\(^{2+}\) keep the CaR in an equilibrium between active and inactive conformations. That is, PO\(_4\)\(^{3-}\) ions enhance the inactive state, while L-amino acids close the cleft in the VFT domain and Ca\(^{2+}\) ions stabilise the active state of the receptor.

The heptahelical domain is the characteristic part of all GPCRs (Goodman, 2004; Alexander et al., 2017) and it consists of 250 amino acids including the seven spanning helices (Brown, 2007). The transmembrane domain contains the likely binding site for the calcimimetic allosteric modulators (Tfelt-Hansen and Brown, 2005).

The intracellular C-terminal domain has five protein kinase C (PKC) phosphorylation sites which play an important role in the negative feedback activity of CaR. Particularly, the PKC-mediated phosphorylation of CaR\(^{T888}\) inhibits the PLC pathway thus decreasing CaR-induced Ca\(^{2+}\) mobilisation (Bai et al., 1998; Davies et al., 2007). Also, the ICD is essential for directing CaR signalling as it contains the heterotrimeric G-protein binding regions in the second and third intracellular loops in its C-terminal domain (Conigrave and Ward, 2013). However, the precise nature of the CaR-induced signaling response depends on two factors; namely the ligand
type and the cell-type in which the CaRs are present (Leach et al., 2014).

### 1.6.2 CaR pharmacology

Although Ca\(^{2+}\) is the primary agonist for CaR, there are many orthosteric type I agonists which mimic the effect of Ca\(^{2+}\) such as Mg\(^{2+}\), Gd\(^{3+}\), polyamines (spermine > spermidine > putrescine; (Quinn et al., 1997) and the aminoglycoside antibiotics including gentamycin and neomycin (Ward and Riccardi, 2012). Type I agonists activate CaR regardless of the presence of Ca\(^{2+}\) whereas type II agonists, such as the calcimimetics, act as allosteric modulators which increase the sensitivity of CaR to Ca\(^{2+}\) (Tfelt-Hansen and Brown, 2005; Nemeth and Goodman, 2016). Some type II agonists are produced normally in our body such as L-amino acids, glutathione, ionic strength and alkaline pH (Ward and Riccardi, 2012) as detailed at the end of this section. L-amino acids bind to the ECD of CaRs as demonstrated by the fact that mutation of three consecutive serines at positions 169, 170 and 171 completely blocks the action of L-Phe as an allosteric modulator (Tfelt-Hansen and Brown, 2005). The first calcimimetics were fendillin-based phenylalkylamines, NPS-467 and NPS-568, these calcimimetics opened the way for the development of the biologically active drug Cinacalcet HCl (Cavanaugh et al., 2012).

Cinacalcet HCl (Mimpara in the EU) (Cavanaugh et al., 2012) has been used successfully as a treatment of secondary hyperparathyroidism associated with end stage renal disease (ESRD) and to control serum calcium levels in primary hyperparathyroidism (Conigrave and Ward, 2013; Nemeth and Shoback, 2013). Calcimimetics bind to the TMD of CaRs stabilising it in an active state and thus decreasing PTH secretion (Cavanaugh et al., 2012). Cinacalcet treatment in patients with ESRD i) lowers PTH secretion after 2-4 h, before it goes up again gradually until 12 h after drug intake (Nemeth et al., 2004) and ii) helps to control plasma PO\(_4\) and Ca\(^{2+}\) since their disturbance is associated with increased risk of cardiovascular mortality and morbidity (Ward and Riccardi, 2012). Also, in a large cohort study conducted to see the effect of calcimimetic treatment on hemodialysis patients, calcimimetic improved all-cause and cardiovascular survival (Block et al., 2010). In addition, calcimimetics proved to delay both calcification of smooth muscle cells *in vitro* (Alam et al., 2008) and atherosclerosis that occurs in apolipoprotein E-deficient mice (Ivanovski et al., 2009). The relative success of Cinacalcet as a therapeutic
agent led to the development of a new CaR allosteric agonist named Benzothiazole which is more potent at lowering PTH and Ca$^{2+}$o than phenylalkylamine calcimimetics (Ma et al., 2011).

Calcilytics oppose the action of Ca$^{2+}$o on CaR and thus enhance endogenous PTH release (Nemeth, 2002; Goodman, 2004; Kumar and Thompson, 2011). Calcilytics have been tested for the possible treatment of osteoporosis because acute intermittent release of PTH has an anabolic effect on bone and thus increases bone density (Ward and Riccardi, 2012). The first calcilytic agents included the phenyl-O-alkylamine NPS-2143 and NPS-89636, which were developed to substitute the subcutaneous injection of PTH in the treatment of osteoporosis (Conigrave and Ward, 2013). However, the clinical results with NPS-2143 were unsatisfactory because it stimulated the catabolic effect of PTH rather than the anabolic effect by inhibiting osteoblast function and differentiation (Gowen et al., 2000; Khan and Conigrave, 2010). This suggested that calcilytics could be more useful in treatment of CaR gain of function mutations as in autosomal dominant hypocalcaemia (Conigrave and Ward, 2013). Recently, an alternate negative allosteric modulator named JTT-305 (MK-5442) was tested by Novartis however it also failed to show a significant increase in bone mineral density (BMD) in postmenopausal women and so was also stopped during its phase III clinical trial (Schwarz et al., 2014).

Moreover, changes in pH and ionic strength also affect the sensitivity of CaRs to Ca$^{2+}$o. Alkalinity enhances the CaR’s sensitivity to calcium while acidity decreases it. This suggests that CaR might have pH-sensing properties in compartments subjected to significant pH variation such as medullary collecting tubules (MCT) in the kidney where the CaR is present in the apical membrane of tubular epithelial cells and in gastric mucosa where it expressed in the basolateral membrane of gastric mucosal cells (Cheng et al., 1999; Busque et al., 2005; Riccardi and Brown, 2010). Also, decreased ionic strength increases the sensitivity of CaR to Ca$^{2+}$o which might help explain the abundance of CaR in the subfornical organ (thirst centre) of the brain (Khan and Conigrave, 2010).
1.6.3 Diseases resulting from CaR mutation and dysfunction

The importance of CaR in the parathyroid gland was confirmed by studies of genetic diseases resulting from either loss-of-function or gain-of-function mutations in the CaR (Tfelt-Hansen and Brown, 2005). Loss-of-function mutations (decreased CaR activity) cause hypercalcaemic disorders, whereas gain-of-function mutations (increased CaR activity) cause hypocalcaemic disorders (Table 1.2).

<table>
<thead>
<tr>
<th>Status of CaSR function</th>
<th>Type of mutation</th>
<th>Disease resulted from the mutation</th>
<th>Manifestation of the disease</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Loss-of-function</strong></td>
<td>Heterozygous (present in one allele only)</td>
<td>Familial hypocalciuric hypercalcemia (FHH)</td>
<td>Hypercalcemia (sometimes asymptomatic), hypermagnesaemia, normal-high PTH level, low urinary Ca$^{2+}$ excretion</td>
<td>(Hebert et al., 1997; Tfelt-Hansen and Brown, 2005; Bilezikian et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>Homozygous</td>
<td>Neonatal severe primary hyperparathyroidism (NSHPT)</td>
<td>Severe Hypercalcaemia, bone demineralisation, multiple fractures and rib cage deformity. Often lethal without early parathyroidectomy</td>
<td>(Tfelt-Hansen and Brown, 2005; Bilezikian et al., 2016)</td>
</tr>
<tr>
<td><strong>Gain-of-function</strong></td>
<td>Heterozygous</td>
<td>Autosomal dominant hypocalcaemia (ADH)</td>
<td>Hypocalcaemia (sometimes asymptomatic), hyperphosphataemia, hypomagnesaemia Nephrocalcinosis, Bartter’s syndrome</td>
<td>(Tfelt-Hansen and Brown, 2005; Mantovani et al., 2017)</td>
</tr>
</tbody>
</table>

Table 1.1. Diseases resulting from mutation of the calcium-sensing receptor

1.6.4 CaR signalling

CaR activation stimulates multiple heterotrimeric guanine nucleotide-binding proteins (G-protein) including Ga$_{q/11}$, Ga$_{i}$, Ga$_{s}$ and Ga$_{12/13}$ to start further downstream intracellular signalling pathways (Conigrave and Ward, 2013; Leach et al., 2014). CaR might also indirectly activate small G proteins such as Rho and Ras (McCudden et al., 2005; Conigrave and Ward, 2013; Leach et al., 2014).
Particularly, in parathyroid gland, CaR is known to activate $\alpha_{q/11}$ and $\alpha_i$-mediated pathways (Conigrave, 2016). Activation of $\alpha_{q/11}$ mediates PTH secretion inhibition via the activation of PLC. The PLC hydrolyses the PIP$_2$ into IP$_3$ and DAG. Subsequently, IP$_3$ and DAG cause the release of Ca$^{2+}$ from endoplasmic reticulum (ER) and activate PKC respectively (Hannan et al., 2016). It should be noted that a slight increase in Ca$^{2+}$ promotes more IP$_3$R opening, while higher Ca$^{2+}$ concentrations inhibit it and this might explain the basis of Ca$^{2+}$ oscillations (Bootman et al., 2001). It has also been reported that CaR phosphorylation status plays an important role in Ca$^{2+}$ oscillations. That is, PKC phosphorylation at threonine-888 in the ICD of CaR inhibits CaR-induced Ca$^{2+}$ mobilisation, whereas dephosphorylation of Thr$^{888}$ enhances Ca$^{2+}$ mobilisation (Conigrave and Ward, 2013). It has been reported by Davies et al. (2007) that CaR$^{T888}$ could undergo rapid dephosphorylation by the action of calyculin-sensitive phosphatase most probably PP2A. The alternative cycle between phosphorylation and dephosphorylation represents another explanation for Ca$^{2+}$ oscillations. The importance of $\alpha_{q/11}$ signalling on PTH regulation has been demonstrated in two studies conducted in mice and another one conducted in man. In a murine study, it has been shown that $\alpha_q$ knockout in PTG increased PTH secretion which subsequently raised blood calcium levels (Wettschureck et al., 2007). In man study, it has been demonstrated that $\alpha_{11}$ loss-of-function mutation caused FHH, while $\alpha_{11}$ gain-of-function mutation caused condition like ADH (Nesbit et al., 2013).

In PTG, CaR activation also decreased cAMP levels possibly by activating the pertussis toxin-sensitive $\alpha_s$-mediated signalling pathway (Chen et al., 1989) or via another $\alpha_s$ independent mechanism (Brown and MacLeod, 2001). It has been reported by Conigrave and Ward (2013) that CaR-induced Ca$^{2+}$ mobilisation could activate phosphodiesterase (PDE)-1 which subsequently inhibits cAMP formation. Interestingly, it has been demonstrated that in CaR-HEK cells, $\alpha_s$-agonists such as forskolin could enhance CaR-induced Ca$^{2+}$ mobilisation (Gerbino et al., 2005; Campion, 2013). These observations strongly suggest that there is a significant crosstalk between the $\alpha_s$ and the $\alpha_{q/11}$-mediated intracellular signalling pathways which will be further investigated in this project.

CaR could also stimulate the $\alpha_s$-mediated intracellular signalling pathway as seen in breast cancer cells that release PT-related protein (PTHrP) (Mamillapalli et al., 2005).
2008), however in normal breast tissue, CaR tends to activate the Gαi-mediated signalling pathway. This suggests that the cell-type in which CaR is expressed could determine the resulting CaR-mediated signalling.

Also, it has been reported that in CaR-HEK cells, CaR could activate Ga12/13 protein which is usually associated with the activation of the monomeric G protein (Rho) (Davies et al., 2006). It has been reported by Burridge and Wennerberg (2004) that Ga12/13-mediated Rho activation is involved in cell cytoskeleton changes. Moreover, Davies et al. (2006) reported that CaR activation with high Ca²⁺, Mg²⁺ or calcimimetic NPS-467 induced actin filament polymerisation in CaR-HEK cells and that the Rho inhibitors Y-27632 and H1152 blocked this effect.

Also, CaR activation has been shown to activate several mitogen-activated protein kinases (MAPKs) including the extracellular signal-regulated kinases (ERK1/2), p38 MAPK, c-Jun NH2-terminal, stress-activated kinase (JNK), P70 S6 kinase, IκBα and the IGFR (Kifor et al., 2001; Tfelt-Hansen et al., 2003; Brennan and Conigrave, 2009; Bin-Khayat, 2016). Kifor et al. (2001) who first reported that Ca²⁺o-mediated CaR activation in bovine PT cells and in CaR-HEK cells enhances ERK1/2 phosphorylation. It is well established that ERK is downstream of Gaq/11-dependent PKC activation and Gai/o (Ward, 2004). That is, in CaR-HEK cells pretreatment with either PTx, the PLCβ inhibitor U73122, or, the PKC inhibitor GF109203X attenuated the Ca²⁺o-induced ERK phosphorylation (Kifor et al., 2001; Ward, 2004). Interestingly, it has been reported by Campion (2013) that in CaR-HEK cells, increasing cAMP levels via the Gaₐ-agonist (forskolin) significantly enhanced CaR-induced ERK phosphorylation. This observation suggests the coexistence of crosstalk between these two signalling pathways.

1.6.5 Signal bias and CaR

CaR can be activated by several agonists including orthosteric and allosteric ligands. Each ligand enables CaR to couple to a specific intracellular signalling pathway and this is what ligand-directed signalling means (Leach et al., 2014). Numerous studies have revealed that the CaR could exhibit ligand directed trafficking or stimulus bias. For example, Thomsen et al. (2012) reported that in HEK293 cells, Ca²⁺o-dependent CaR activation preferentially stimulates Gaq and Ca²⁺i mobilisation over ERK1/2
phosphorylation however, the allosteric agonist (spermine) favours CaR-mediated ERK1/2 phosphorylation. Moreover, Rey et al. (2010) reported that CaR activation with Ca\(^{2+}\), mediates Ca\(^{2+}\)\(_i\) mobilisation via activation of Ga\(_{q/11}\)/PLC signalling pathway, whereas L-phenylalanine (L-Phe) tends to produce low frequency Ca\(^{2+}\)\(_i\) oscillations via the activation of Ga\(_{12/13}\) and transient receptor potential channel-1 (TRPC1) which allowed the influx of Ca\(^{2+}\)\(_o\) from extracellular fluid (Rey et al., 2005; Rey et al., 2006; Leach et al., 2014). Ligand-biased signalling has the potential to permit pharmacologist to discover new drugs that favourably activate one pathway over another to produce distinct action with fewer side effects.

![Diagram of biased signalling and CaR](image)

**Figure 1.4. Biased signalling and CaR**

In ligand bias, both agonists A and B activate CaR through stimulation of 3 different signalling pathways. However, agonist A favourably gives effect A and agonist B favourably gives effect B.
1.7 Potential Gαs-coupled GPCRs signalling involved in the regulation of PTH secretion

1.7.1 Prostanoid receptors

1.7.1.1 Prostanoid synthesis and function
Prostanoids include prostaglandins (PGs) and thromboxanes (TXs) (Sugimoto et al., 2000). The prostanoid biosynthesis pathway involves cyclooxygenase enzyme (COX) which converts arachidonic acid first to prostaglandin endoperoxide (PGG₂), then into PGH₂. Subsequently, PGH₂ is transformed into different PGs and TXs including PGD₂, PGE₂, PGF₂α, PGI₂ and TXA₂ via the action of different prostanoid synthases (Sugimoto and Narumiya, 2007; Cornejo-García et al., 2016) (Figure 1.4). There are two isoforms of COX enzyme in human; COX-1 which is constitutively expressed in almost all cell types and COX-2 which is transiently induced by inflammatory mediators (Caughey et al., 2001; Claar et al., 2015). The non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin and indomethacin inhibit COX and thus decrease prostanoid production (Sugimoto and Narumiya, 2007). Prostanoids play an important role in various physiological processes in the body including; contraction and relaxation of vascular and respiratory smooth muscles, neurotransmitter secretion in fever and sleep, gastrointestinal tract motility and secretion, water and ion transportation in the kidney, cell apoptosis and differentiation and platelet aggregation (Breyer and Breyer, 2000; Sugimoto and Narumiya, 2007).

1.7.1.2 Prostanoid receptors signalling
Prostanoids exert their function via activation of distinct G protein-coupled receptors which then change the level of the second messenger inside the cells (Sugimoto et al., 2000). The prostanoid PGD₂ binds to either DP₁ or DP₂ receptors whose activation then leads to either increased or decreased cAMP accumulation via activation or inhibition of the AC enzyme respectively (Claar et al., 2015). The prostanoid PGE₂ can bind to four EP receptor subtypes (1-4). The EP₁R couples to Gαq/11 protein and thus increases Ca²⁺; concentrations (Alexander et al., 2017). Signalling through EP₂ and EP₄ receptors mainly increases cAMP levels via coupling to Gαs protein however the EP₄R can also couples to the Gαi which can
activate PI3K/ERK signalling (Konya et al., 2013). On the other hand, EP₃R couples to Gaᵢ protein with a resultant decrease in cAMP concentrations inside the cell (Carboneau et al., 2017). PGF₂α stimulates FP receptor which couples to Ga₉/₁₁ protein and thus increases Ca²⁺ᵢ levels (Hata and Breyer, 2004). The prostacyclin PGI₂ activates the Gaᵦₕ-mediated intracellular signalling pathway and thus increases intracellular cAMP levels (Carboneau et al., 2017) (Figure 1.4).

### 1.7.1.3 Prostanoid receptors pharmacology

The prostaglandins play various roles in normal homeostasis and inflammatory diseases (Cornejo-Garcia et al., 2016). They have very short biological half-lives from seconds to a few minutes, however the discoveries of highly selective prostaglandin agonists and antagonists have facilitated the determination of the distinct functions of each prostaglandin receptors and sometimes the overlapping roles for them (Konya et al., 2013). The benefit of having such selective drugs could minimise the side effects from using the classical COX inhibitors drugs.

Prostaglandins receptors might exhibit stimulus bias that is, both ligand and cell type could direct the intracellular signalling pathway being activated. For example, in HEK293 cells transfected with EP₄R, PGE₂ activates Gaᵦₕ-mediated signalling however, the EP₄R agonist L-902688 and PGE₁-OH biased the signalling towards Gaᵢ and β–arrestin pathway (Konya et al., 2013).

Prostaglandin’s agonists and antagonists are commonly used as potential treatments in different pathological conditions and here is a list of some examples: i) the selective EP₃R agonist PF-04217329 effectively reduces the ocular pressure in open angle glaucoma patients (Prasanna et al., 2011) ii) the selective EP₃R antagonist TG4-155 significantly lowers the neuronal injury in hippocampus when administered 1 hour after inducing status epilepticus in mice (Jiang et al., 2012) iii) administration of the selective EP₄R agonist ONO-AE1-329 strongly protects against hepatic ischemic injury in comparison with other selective EP receptors agonists (Kuzumoto et al., 2005).

### 1.7.1.4 Prostanoids in parathyroid gland

Early in 1985, Brown and Swartz mentioned that dispersed bovine PT cells produced prostaglandins including; PGF₁₆α, PGF₂α, PGE₂ and TX₂β₂ (Brown and Swartz, 1985).
Also, it has been reported that in dispersed bovine PT cells, PGE\textsubscript{2} increased cAMP levels and subsequently enhanced the PTH release (Gardner et al., 1978; Gardner et al., 1980). Recently in 2015, it has been reported that the COX-2 inhibitor NS398 significantly decreased the PTH release from perifused human PT cells exposed to low Ca\textsuperscript{2+} concentration. It has been also found that the non-selective prostanoid inhibitor AH6809, the selective EP\textsubscript{4}R and the selective IPR antagonists suppressed PTH release acutely and reversibly (Szczawinska et al., 2015). In conclusion, the prostanoid dependent intracellular signalling pathways could play a role in the intrinsic mechanism of PTH secretion.

![Summary of prostaglandins biosynthesis and intracellular signalling](image)

Figure 1.5. Summary of prostaglandins biosynthesis and intracellular signalling

*Adapted from* (Konya et al., 2013)
1.7.2 Histamine receptors

1.7.2.1 Histamine synthesis and function
Histamine is synthesised from the amino acid L-histidine by the action of histidine decarboxylase (HDC) enzyme in histamine-producing cells (Bahre and Kaever, 2017; He et al., 2017). Histamine can either be stored in intracellular granules or spontaneously released as in epithelial cells or lymphocytes. The synthesis and metabolism of histamine are tightly controlled to avoid unwanted reactions upon its activation as it exerts potent effects at very low concentration (Bahre and Kaever, 2017).

Histamine plays an important role in multiple physiological and pathological conditions including; vasodilation and increased vascular permeability in allergic reaction, contraction of smooth muscle cells in airways, uterus and intestine, increase heart rate, acid secretion in stomach, neurotransmission, hematopoiesis, cancer cell proliferation and immunomodulation (Maintz and Novak, 2007).

1.7.2.2 Histamine receptors signalling
Histamine acts on multiple histamine receptors including H₁R, H₂R, H₃R and H₄R. H₁R primarily couples to Gα₁₁ protein which then activates PLC-mediated intracellular pathway causing IP₃ formation and Ca²⁺ mobilisation (Panula et al., 2015). H₂R preferentially couples to Gαs-protein activating AC and increases cAMP production (Alexander et al., 2017). Conversely, H₃R and H₄R activation decreases cAMP level via activation of the pertussis toxin-sensitive Gαi-protein (Schneider et al., 2009) (Figure 1.5).
1.7.2.3 Histamine signalling in parathyroid gland

The presence of (histamine-releasing) mast cells in parathyroid glands was noted first by Erdheim (1903) and was mentioned as a part of normal parathyroid gland histology by Morgan and Gilmour (Morgan, 1936; Gilmour, 1939). Moreover, it has been noted that mast cells are significantly higher in adenomatous or hyperplastic parathyroid tissue compared to normal tissue and that the mast cells tend to be in the peripheries of the diseased glands rather than the centre as in normal tissues (Anderson, 1974).

Sherwood et al. (1980) found that the H₂R blocker cimetidine (Tagamet; 300mgs four times daily) decreased PTH levels to normal and improved the signs and symptoms in patients with hyperparathyroidism. However, the reduction of \(\text{Ca}^{2+}_o\) concentration was dependent on the dose, time and the route of cimetidine administration. Also, he found that after stopping cimetidine, a rebound increase in PTH level occurred and according to that he suggested that cimetidine either blocks the synthesis or the secretion of PTH (Sherwood et al., 1980).

Abboud et al. (1981) stated that diseased parathyroid glands (adenomatous or hyperplastic) contain high levels of hisatmine as measured by radioimmunoassay.

Figure 1.6. Summary of histamine biosynthesis and intracellular signalling
addition, it has been reported by Williams et al. (1981) that in vitro, histamine enhanced iPTH secretion from PT cells and that effect was antagonised by the H\textsubscript{2}R blocker cimetidine. Also in the same article, it was reported that in normal subjects, cimetidine induced PTH secretion inhibition suggesting that endogenous histamine might modulate PTH secretion. In 1981, Sicard et al reported that in hyperparathyroid cell preparations, histamine stimulated cAMP production and that cimetidine inhibited the histamine-mediated cAMP production at low calcium concentrations (0.5 mM) (Sicard et al., 1981).

1.7.3 B-adrenergic receptors

Adrenoceptors including \(\alpha\) and \(\beta\) subtypes are from class A rhodopsin-like GPCRs (Lee et al., 2017). There are three subtypes of \(\beta\) adrenoceptors; the \(\beta_1\), \(\beta_2\) and \(\beta_3\) (Alexander et al., 2017). All three subtypes couple to the \(G_{\alpha_s}\)-protein activating AC and increase cAMP production (Bylund et al., 1994), however it has been reported that \(\beta_2\) adrenoceptors could couple to the \(G_{\alpha_i}\) protein to balance the effect of \(G_{\alpha_s}\) on cAMP production in some cardiovascular disorders (Spadari et al., 2017). The \(\beta_1\) adrenoceptors are widely-distributed in cardiovascular and adipose tissues while the \(\beta_2\) adrenoceptors are responsible mainly for relaxation of smooth muscle cells in vessels, airways and uterus (Bylund et al., 1994). On the other hand, the \(\beta_3\) adrenoceptors have been shown to have effects on lipolysis, thermogenesis and relaxation of the urinary bladder (Sawa and Harada, 2006). The endogenous agonists; noradrenaline and adrenaline activate all \(\beta\) adrenoceptors with different potencies e.g. \(\beta_2\) adrenoceptors are more sensitive to adrenaline, while \(\beta_3\) adrenoceptors respond to both adrenaline and noradrenaline equally (Bylund et al., 1994; Alexander et al., 2017). Numerous agonists and antagonists for \(\beta\) adrenoceptors have been developed and they are commonly used in many pathological conditions. For example, the \(\beta_1\) adrenoceptor antagonist atenolol, the \(\beta_2\) adrenoceptor agonist salbutamol and the \(\beta_3\)-adrenergic agonist mirabegron are commonly used to treat hypertension, asthma and overactive bladder syndrome respectively (Alexander et al., 2017).

Early in 1977, it has been reported that low calcium concentrations as well as \(\beta\)-adrenergic agonists in order of potency (isoproterenol > adrenaline > noradrenaline) stimulate cAMP production and PTH release in bovine parathyroid cells. However,
the effect of low calcium concentrations in cAMP accumulation was only 3% of that
obtained with isoproterenol suggested that factors other than low calcium
concentrations may contribute in PTH release (Brown et al., 1977b).

1.7.4 Calcitonin receptor-like receptor (CALCRL)

The CALCRL makes CGRP, AM1 or AM2 when it combines with RAMP-1,
RAMP-2 or RAMP-3 respectively (Conner et al., 2005). All CGRP, AM1 and AM2
are commonly coupled to the $\text{G}_{\alpha_s}$-protein activating AC and thus they increase
cAMP accumulation (Alexander et al., 2017). The endogenous agonists for CGRP
are $\alpha$CGRP and adrenomedullin with $\alpha$CGRP being more potent as a ligand. In
contrast, AM1 receptor preferentially binds to adrenomedullin, while AM2 can bind
to both CGRP and adrenomedullin with equal potency (Russell et al., 2014;
Alexander et al., 2017). The calcitonin family peptides are widely expressed
throughout the body especially in the peripheral and central nervous system (Walker
et al., 2010). CGRP and adrenomedullin are known potent vasodilators as well as
CGRP has been linked to inflammation and nociception (Durham and Vause, 2010).

In parathyroid gland, plasma adrenomedullin levels have been reported to be raised
in patients with primary hyperparathyroidism (PHP) (Letizia et al., 2004) with
adrenomedullin immunoreactivity found in nearly two-thirds of hyperplastic and
adenomatous parathyroid gland cells. Moreover, AM22-52 a selective
Adrenomedullin receptor antagonist elicits increased PTH secretion in preliminary
functional experiments from the lab of our collaborator Professor Arthur Conigrave
suggesting that adrenomedullin may have an autocrine modulatory effect in
suppressing PTH secretion (unpublished data).

1.8 Aims and objectives

The CaR-regulated secretion of PTH is the main controller of serum calcium
concentration. As detailed in this chapter, the negative feedback mechanism involved
in the inhibition of PTH secretion by CaR is relatively well understood, whereas in
contrast, little is known about what drives PTH secretion when CaR inhibition is
removed. Thus, the principle objectives of my project were:
**Objective 1:** To identify genes and proteins of certain G\(\alpha\)-coupled GPCRs (and the enzyme(s) responsible for the local production of their ligand) in bovine PTG that could potentially represent the intrinsic driving force for cAMP generation and PTH secretion (Chapter 3).

**Objective 2:** To determine the effect of increasing intracellular cAMP on CaR-induced Ca\(^{2+}\) mobilisation at subthreshold Ca\(^{2+}\)\(_o\) concentrations and to determine whether the calcilytic NPS-2143 inhibits this effect in CaR-HEK cells (Chapter 4).

**Objective 3:** To determine the effect of several G\(\alpha\)-agonists including; PGE\(_2\), isoprenaline and histamine on CaR-induced Ca\(^{2+}\)\(_i\) mobilisation both on their own and then in combination, and, the effect of PGE\(_2\) on ERK1/2 and P70 S6 kinase phosphorylation in CaR-HEK cells (Chapter 4).

**Objective 4:** To determine the effect of CaR stimulation on PGE\(_2\)-induced cAMP production in CaR-HEK cells and to identify the G-protein being activated upon CaR stimulation using a FRET-based Epac sensor (Chapter 5).

**Objective 5:** To determine whether the calcilytic NPS-2143 enhances or antagonises the effect of \(\beta_2\)AR agonist salbutamol on Ca\(^{2+}\)\(_i\) mobilisation and ERK1/2 activation in BSMCs (Chapter 5).
CHAPTER 2

Materials & Methods
2.1 Materials

HEK-293 cells were stably transfected with the human CaR, a gift from Dr E. F. Nemeth (NPS Pharmaceuticals, Bedminster Township, NJ, USA). The cAMP FRET reporter was provided from Jeffrey Klarenbeek (NKI-AVL, Amsterdam). Dulbecco’s modified Eagle’s medium, phosphate-buffered saline (PBS), trypsin-EDTA solution and Foetal bovine serum (FBS) as well as acrylamide/bis-acrylamide (40% solution) were purchased from Sigma-Aldrich (Gillingham, UK). Nitrocellulose blotting membrane (0.45 µM) was purchased from GE Healthcare Life Sciences (Buckinghamshire, UK) and the enhanced chemiluminescence solution (ECL) was from Bio-Rad Laboratories Ltd. (Watford, UK). All primary antibodies (phospho-PKA substrate, phospho ERK, phospho P70 S6 kinase and β-actin) as well as horse radish peroxidise-conjugated secondary anti-mouse antibody were ordered from Cell Signalling Technology Inc. (Leiden, The Netherlands). EP₄R and CALCRL primary antibodies were from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Secondary polyclonal goat anti-rabbit antibody was purchased from Dako (Agilent Technologies Denmark ApS.). EP₄R, IPR and CALCRL plasmids (pcDNA3.1+) were ordered from cDNA Resource Centre (Bloomburg, PA, USA). XL-1 blue competent cells were from Agilent Technologies LDA UK Limited (Stockport, Cheshire, UK). S. O. C. medium was from Invitrogen by life technologies (Altrincham, UK). FuGENE 6 HD Transfection Reagent was purchased from Promega (Southampton, UK). Fura-2, AM used to visualise the intracellular calcium and blot stripping buffer were purchased from Thermo Fisher Scientific (Altrincham, UK). BioScript Reverse Transcriptase, Biotaq DNA polymerase and HyperLadder 100 bp were from Bioline (Humber Road, London, UK). All primers were from Sigma-Aldrich (Gillingham, UK). Safe View Nucleic acid stain was obtained from nbs biologicals (Huntingdon, Cambridgeshire, UK). PNGase F and DNase I (RNase-free) were from New England Biolabs (Hitchin, UK). The PTGER4/EP₄ primary antibody used in immunofluorescence was purchased from Thermo Fisher Scientific (Altrincham, UK), and the Alexa Fluor 488 goat anti-rabbit secondary antibody was from Invitrogen (Altrincham, UK). Prolong gold antifade reagent with DAPI was ordered from Molecular Probes (Altrincham, UK). Various signalling reagents including prostaglandin E₂, human calcitonin gene-related peptide (CGRP), isoprenaline hydrochloride, atenolol, histamine dihydrochloride, pyrilamine,
ranitidine and pertussis toxin were purchased from Sigma-Aldrich (Gillingham, UK). All (selective EP4 receptor antagonist L-161,982, calcilytic NPS-2143, non-selective prostaglandin receptors antagonist AH 6809) were purchased from Tocris Bioscience (Bristol, UK). PKA inhibitor H89 was purchased from Cayman Chemical (Cambridge Bioscience Ltd., Cambridge, UK).

2.2 Bovine parathyroid cell membrane lysate preparation

The bovine parathyroid glands were isolated from less than thirty-month-old freshly-killed cattle. The glands were kept in pH 7.4 buffer containing 141 mM NaCl, 5.3 mM KCl, 20 mM HEPES, 5 mM MgSO4 and 2 mM CaCl2. The glands were kept on ice during transportation then stored in a -80ºC freezer. The particulate fractions were prepared by homogenising the glands using a tissue grinder in around 10 ml homogenisation buffer. The homogenisation buffer contained 12 mM HEPES (pH 7.6), 300 mM mannitol supplemented with 1.25 µM pepstatin, 4 µM leupeptin, 4.8 µM PMSF, 1 mM EDTA, 1 mM EGTA and 1 mM N-ethylmaleimide (NEM). Then, each homogenate was centrifuged at 2,500 g for 10 minutes at 4ºC. After centrifugation, the postnuclear supernatant was taken and ultra-centrifuged at 100,000 g for 30 minutes at 4ºC. The membrane lysates were prepared by resuspending the formed pellets in 500 µl of same homogenisation buffer then stored at -80ºC for further use.

2.3 Immunoblotting

The immunoblotting was performed using 7.5, 8 or 10% SDS-polyacrylamide gels to separate the proteins on the basis of molecular weight. The samples were diluted in 4:1 ratio 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) bromphenol blue and 5% (v/v) β-mercaptoethanol reducing agent. Where heat denaturation was necessary, the samples were heated at 85ºC in a heating block for 3 minutes then loaded into the previously prepared SDS-polyacrylamide gels. Then, the proteins bands were separated by placing the gel cassette in a tank containing running buffer comprising of 25 mM Tris, 192 mM glycine and 0.1% SDS. The tank was connected to a power supply at 84 mA for 1.5-2 hours. Next, proteins in the gel were transferred
electrophoretically onto nitrocellulose membrane (pore size 0.45 µm) over 1 hour at 200 mA in blotting buffer containing 25 mM Tris, 200 mM glycine and 15% (v/v) methanol. The membrane was incubated in a blocking buffer solution containing Tween-TBS solution (15 mM Tris [pH 8.0], 150 mM NaCl, 0.1% (v/v) Tween 20) and 5% (w/v) bovine serum albumin (BSA) to block non-specific binding sites. Then, the membrane was probed with the primary antibody (1:2000 -1:5000) for 1 hour followed by 30 minutes washing using Tween-TBS with the buffer changed every 10-15 minutes. Afterwards, the membrane was probed with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour followed by 3 times (10 minutes) washing with Tween-TBS. Finally, BIO-RAD clarity western ECL substrate and Kodak MR Film were used to visualise the membrane. Immunoreactive bands were analysed semi-quantitatively by densitometry (Ward et al., 1998).

If protein deglycosylation was required, PNGase F kit was used following manufacturer's instructions. Briefly, 2 µl of 10X glycoprotein denaturing buffer and 20 µl of bovine parathyroid membrane lysates were heated at 100°C for 10 minutes. Then, 4 µl of 10X G7 reaction buffer, 4 µl of 10% NP-40, 2 µl of PNGaseF and 8 µl of H2O were added to the mixture to make the total volume of 40 µl. Next, the mixture was incubated at 37°C for 1 hour. Lastly, the sample was diluted in 4:1 ratio with 5X Laemmli buffer and loaded in SDS-polyacrylamide gel for western blotting.

2.4 Immunoblot stripping

Using Restore western blot stripping buffer, antibodies attached to the nitrocellulose membrane were stripped off following manufacturer's instructions. Simply, the membrane was incubated in stripping buffer at 37°C for 15 minutes with intermittent agitation. Next, the membrane was washed from the stripping buffer over the shaker in Tween TBS for 30 minutes (3 x 10 minutes). Finally, the membrane was re-blocked in 5% BSA and then processed for immunoblotting using a different antibody, as before.
2.5 DNA transformation into competent cells

5 µg of dried plasmid (EP$_4$R or CALCRL) were mixed with 500 µl of water to make the final concentration 10 ng/µl. The plasmids (pcDNA3.1+) were then transformed into XL1-Blue Competent cells following the manufacturer’s instruction. Briefly, 10 ng of each plasmid was added to different XL1-Blue cells and chilled on ice for 30 minutes. Cells were then heat-shocked at 42°C for 45 seconds before being chilled again on ice for 2 minutes. 900 µl of preheated SOC medium was added and then the solution was incubated at 37°C for 1 hour. The transformed bacteria were then spread on agar plates containing ampicillin (100 mg/ml) and left overnight in a 37°C incubator. Then, two colonies were selected and grown in 50 ml LB-broth containing ampicillin and left overnight on an orbital shaker at 37°C. A Qiagen Plasmid Midi kit was used to elute the DNA in accordance with manufacturer’s instructions. The DNA yield was determined by measuring the DNA concentration using a Nano-drop spectrophotometer and by examining the quality of the DNA in an agarose gel.

2.6 Cell culture

Dulbecco’s modified Eagle’s medium (DMEM) containing 10% foetal bovine serum (FBS) was used to maintain CaR-HEK cell growth. Cultured cells were grown to 70-80% confluence then, the old media in the flask was removed and the cells were washed once with phosphate-buffered saline (PBS). Next, 2 ml of trypsin was added and kept for 3 minutes at 37°C to disassociate the cells. Afterwards, 5 ml of new serum-containing media was mixed gently with the cells and trypsin. The cell suspension was then centrifuged at 2,000 g for 5 minutes with the formed pellet then resuspended in 10 ml serum-containing media. 1-3 ml of the resuspension was added to a new serum-containing media in a flask and maintained at 37°C in an incubator with 5% CO$_2$ atmosphere. The cells were not used for more than 30 passages after transfection (Maldonado-Pérez et al., 2003).

2.7 Transient cell transfection

Transient cell transfection was performed in T25 flasks by adding FuGene6 to 50% confluent cells. In summary, nothing or 1 µg of vector and 3 µl of transfection agent were mixed with 97 µl of serum-free media (total 100 µl) and incubated for 10
minutes at room temperature. Then, each solution was added to different CaR-HEK cells drop by drop and incubated at 37°C. Experiments were performed within 48-72 hours of transfection.

2.8 Cell assay and lysates preparation

Cells were cultured in 35 mm dishes and when they were 80% confluent, the cell assay was performed. First, media was removed and the cells were rinsed quickly with PBS. Then, cells were equilibrated in 0.5 mM CaCl₂-containing buffer for 20 minutes at 37°C before adding the desired treatment for 10 minutes at 37°C. Next, cells were lysed for 10 minutes on ice in 150 µl RIPA buffer supplemented with 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 and 4.8 µM PMSF. Finally, the lysate was collected, centrifuged at 10,000 g for 10 minutes at 4°C and the supernatant then collected and stored at -80°C until use.

2.9 Intracellular calcium imaging

Cells were cultured on 16 mm glass coverslips 24 hours before imaging in DMEM containing 10% FBS. Cells were loaded in the dark with 1 µM fluorescent dye (Fura-2-AM) which is sensitive to changes in calcium concentrations. The Fura-2-AM was mixed with 1.2 mM CaCl₂-containing experimental buffer (see below) supplemented with 0.1% (w/v) BSA (Davies et al., 2006; Absi et al., 2014). Experimental buffer contained 0.5 mM CaCl₂, 20 mM HEPES (pH 7.4), 125 mM NaCl, 0.5 mM MgCl₂, 4 mM KCl and 5.5 mM glucose where the CaCl₂ concentration was progressively increased (up to 10 mM), the NaCl concentration was decreased (to as little as 110 mM) to maintain a consistent ionic strength. Next, the coverslip was placed in a perfusion chamber sited on an inverted microscope connected to a Nikon Diaphot camera. Changes in Ca²⁺ᵢ concentrations in response to changes in Ca²⁺ₒ concentrations were measured as a change in the 350/380 nm ratio by microfluorometry. The baseline for the curves were calculated when cells were
exposed to 0.5 mM Ca$^{2+}$. Metafluor Software was used to take all images and curves and the quantitative analysis was performed using GraphPad Prism software.

2.10 **Total RNA extraction**

The freshly-obtained bovine parathyroid glands were immediately placed in RNA later solution on ice and transported to the laboratory. At the time of RNA extraction, glands were dissected from the surrounding fat tissues then weighed and processed using an RNeasy Mini kit according to the manufacturer’s instructions. In summary, the tissue was homogenised in 1 ml “RLT” buffer mixed with 10 µl β-mercaptoethanol. Then, the lysate was transferred into new micro-centrifuged tubes and kept in room temperature for 5 minutes. Next, the sample was mixed thoroughly with 200 µl chloroform then, centrifuged at 12,000 g for 15 minutes at 4°C. The formed upper aqueous phase was transferred into a new tube and was mixed by vortexing with 1 volume of 70% ethanol. The sample then was transferred into two RNeasy mini spin columns placed in collection tubes and were centrifuged twice at 10,000 g for 15 seconds at room temperature and the flow-through discarded each time. Then, 500 µl of “RPE” buffer was added to each RNeasy spin column and centrifuged for 2 minutes at 10,000 g to wash the membrane. At the end, the RNeasy spin column was transferred into a new collection tube and 40-50 µl of RNA-free water was added after which the column was centrifuged for 1-minute at 10,000 g to elute the RNA. The RNA concentration was measured using a Nano-drop spectrophotometer and the samples were kept in -80 ºC for further use.

2.11 **Reverse transcription polymerase chain reaction (RT-PCR)**

Both BioScript reverse transcriptase and Biotaq DNA polymerase were obtained from the Bioline Company (Humber Road, London, UK). The reverse transcription (RT) reaction was carried out by boiling the RNA sample at 65°C for 10 minutes. Then, on ice 4 µl 5X reverse transcriptase buffer was mixed with 1 µl RNase inhibitor, 2 µg of RNA sample, 1 µl random primers, 2 µl dNTPs mix (10 mM) and then made up to a 20 µl final volume by adding RNase-free water either in the absence or presence of 400U (2 µl) of reverse transcriptase enzyme AMV with the
mixture then incubated at 42°C for 1 hour. The resultant cDNA was stored at 80°C until use for RT-PCR.

Next, the RT-PCR mixture was prepared by mixing 0.5 µl of (10 mM) dNTPs, 1 µM forward primer, 1 µM reverse primer, 250 ng cDNA, 1 U 10X NH₄ reaction buffer, 0.6 U (1.5 µl) 50 mM MgCl₂ solution and 2.5 U Biotaq DNA polymerase then made up to a 25 µl final volume by adding RNase-free water. All RT-PCR mixtures containing specific forward and reverse primers were placed in the PCR machine and run as follows: a) 94°C for 4 minutes (initial denaturation), at 60°C for 2 minutes, at 72°C for 3 minutes followed by b) 40 cycles of 94°C (denaturation), 60°C (annealing) and 72°C (elongation) for 45 seconds each in duration, then c) a final elongation step at 72°C for 10 minutes ending with cooling at 4°C. Primers were designed against bovine EP₄R, PGE synthase 1, PGE synthase 2, CALCRL, RAMP-1, RAMP-2, RAMP-3 and Adrenomedullin (Table 2.1).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction</th>
<th>Sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP₄ receptor</td>
<td>Forward</td>
<td>GCTTGTGGGTGGCGAGTGTTCC</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGAATGGGGCTCACAGAAGC</td>
<td></td>
</tr>
<tr>
<td>PGE synthase 1</td>
<td>Forward</td>
<td>GTGGCATAACCTTGGGAAA ACT</td>
<td>203</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AATCTCAAAGGGCCATCGGT</td>
<td></td>
</tr>
<tr>
<td>PGE synthase 2</td>
<td>Forward</td>
<td>GTTCTCTGTGTCTCCCTGCCCC</td>
<td>181</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAGTCCCAACACAGGAGGGGT</td>
<td></td>
</tr>
<tr>
<td>CALCRL</td>
<td>Forward</td>
<td>TAACCTGACCTACACTCTGCTGCT</td>
<td>282</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTTTCAGTCAGCTACACTGGGG</td>
<td></td>
</tr>
<tr>
<td>RAMP1</td>
<td>Forward</td>
<td>AGTTCCAGGACACACATGGGA</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGGACGACGAGGAAGAACC T</td>
<td></td>
</tr>
<tr>
<td>RAMP2</td>
<td>Forward</td>
<td>CCAGACACGCTCACAAGTGCA</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGATGATCTCCTTCCGCCCAG</td>
<td></td>
</tr>
<tr>
<td>RAMP3</td>
<td>Forward</td>
<td>CAGGCTTGAGTGTCCTCCGTGA</td>
<td>216</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAAAGGCACAGCAGCAGAGAGC</td>
<td></td>
</tr>
<tr>
<td>Adrenomedullin</td>
<td>Forward</td>
<td>CTCTTATCGGCCCCAGGAT</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCTTGTCCCTGTCCGGTA</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1. Primers sequences and RT-PCR products sizes**

for bovine EP₄R, PGE synthase 1, PGE synthase 2, CALCRL, RAMP-1, RAMP-2, RAMP-3 and Adrenomedullin
2.12 Immunofluorescence

Cells were cultured in 16 mm glass coverslips and when they reached 80% confluence, immunofluorescence was carried out. First, media was removed and cells were washed quickly with PBS. Next, cells were treated with different buffers contained 0.5 or 5 mM CaCl$_2$, then cells were fixed with 4% (w/v) paraformaldehyde (PFA) for 15 minutes at room temperature. Then, the cells were washed with PBS 3 times 5 minutes each followed by permeabilisation in 0.5% (w/v) saponin dissolved in PBS for 10 minutes. After removing saponin, cells were washed with PBS 3 times for 5 minutes, then cells were incubated for one hour at room temperature in blocking buffer containing 1% BSA in PBS. Cells were then incubated overnight at 4°C in PBS containing 1% BSA and the anti-EP$_4$R primary rabbit polyclonal antibody (1:200) followed by washing with PBS 3 times for 5 minutes each. Cells were then incubated for one hour with the secondary Alexa Fluor 488 goat anti-rabbit (1:1000) at room temperature in the dark. At the end, cells were washed with PBS 3 times for 5 minutes and the coverslips were mounted on a glass slide in one drop of prolong gold antifade reagent with DAPI.

Images were collected on an Olympus BX51 upright microscope using a 100x / 1.35 UPlan FLN oil objective and captured using a Coolsnap ES2 camera (Photometrics) through Metavue v7.8.4.0 software (Molecular Devices). Specific band pass filter sets for DAPI and FITC were used to prevent bleed through from one channel to the next. Images were then processed and analysed using Fiji ImageJ (http://imagej.net/Fiji/Downloads).

2.13 Fluorescence resonance energy transfer (FRET)

50% confluent CaR-HEK cells were transiently-transfected with the cAMP vector (H187) 48 hours before the experiment as described in section (2.7). Then, 24 hours before the experiment, cells were trypsinised and seeded onto 16 mm glass coverslips along with the Fugene-containing media. At the time of the experiment, the coverslip was placed in a perfusion chamber and images were acquired every 5 seconds over 10 minutes using Olympus IX83 inverted microscope equipped with a 60x/1.42 Plan Apo oil objective. Experimental solutions contained PGE$_2$ (10 nM), G$_{aq}$ inhibitor YM254890 (100 nM), GF102903X (300 nM), R568 (1 µM) or
pertussis toxin (PTx; 100 nM/1 ml) were prepared in 0.5-5 mM Ca\(^{2+}\)-containing buffers. The fluorescence light path consisted of a Lumencor LED light engine using the blue LED filtered to provide Cyan excitation light (BP440/20 nm), a CFP/YFP dual colour dichroic mirror and an emission filter wheel (Prior Scientific) providing specific emission wavelengths for CFP (BP482/25 nm) and YFP (BP544/24 nm). Images were acquired through MetaMorph software (Molecular Devices) using an R6 CCD camera (QImaging). Subsequent image analysis was performed through ImageJ software (http://imagej.net/Fiji/Downloads) using measure stack analysis tool in a region of interest within the cell. FRET was expressed as ratio of CFP/YFP emission intensities. For each condition, the average of the FRET ratio in the last minute of the treatment was calculated. The quantitative analysis was performed using GraphPad Prism software and the data from 6-12 experiments were expressed as means ± standard error of the mean (SEM).

The Epac (H187) construct consists of part of the exchange protein that undergoes a conformational change upon cAMP binding (Epac) fused to two fluorophores in its N- and C-terminal. The donor cyan fluorescent protein mTurquoise2 is fused to the N-terminal of the Epac while the acceptor tandem of the circular pre-mutated cp173 Venus is fused to the C-terminal of the Epac (Klarenbeek et al., 2015; Storch et al., 2017) (Figure 2.1). Raised cAMP levels resulted in increase the distance between the two fluorophores and a decrease in YFP/CFP ratio.
Figure 2.1. Model for the conformational change induced by cAMP binding to the regulatory part of Epac detected by FRET.

Following cAMP binding to the regulatory domain of Epac, the VLVLE sequence interacts with the regulatory domain, exposing the GEF and allowed its binding with the Rap1 to mediate the downstream effector. Increasing the distance between the CFP and YFP permits the detection of this conformational change. CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; DEP, dishevelled/Egl-10/pleckstrin domain; REM, RAS exchange motif; GEF, guanine nucleotide exchange factor; Rap1, RAS-like small GTPase protein. Adapted from (Ponsioen et al., 2004).

2.14 Statistical analysis

Data were analysed using Microsoft Excel and GraphPad Prism (V 7). Data are presented as means ± S. E. M and values of P<0.05 were considered statistically significant. Normality was determined using Shapiro-Wilks test. To determine the EC_{50} and the Hill coefficient, sigmoidal dose-response (variable slope) fitted by GraphPad Prism (V 7) was used. The exact statistical test used for each experiment is described in the individual result section below. n is defined as the number of dishes of cells used for western blotting or the number of individual coverslips used when measuring intracellular calcium and cAMP/FRET.
CHAPTER 3

Identification of Candidate Components of an Intrinsically-Stimulated Parathyroid Hormone Secretion Mechanism
3.1 Introduction

The first objective of this project was to identify $G\alpha$-coupled GPCRs expressed in the bovine parathyroid gland that, by generating cAMP, may positively drive PTH secretion. A number of studies conducted predominantly in the 1980s identified different exogenous agonists capable of stimulating PTH secretion but where a fuller molecular analysis was lacking. Therefore, here I combined microarray data from bovine and murine PT glands with RT-PCR and immunoblotting data to confirm the expression of certain GPCRs in PT gland. It should be noted that this chapter represents the molecular element of a collaborative study being conducted together with Professor Arthur Conigrave (University of Sydney) who was measuring the effects of various GPCR agonists and antagonists on PTH secretion from human PT cells.

3.2 Materials and Methods

Bovine PTG cells were isolated and cultured as described in Section 2.2. Western blotting and RT-PCR were performed as described in Section 2.3 and 2.11 respectively. CaR-HEK cells were cultured as described in section 2.6 and immunofluorescence was performed as described in section 2.12. Intracellular calcium imaging was performed as detailed in section 2.9.

3.3 Results

3.3.1 Expression of GPCRs and related proteins in bovine and murine PT gland as revealed by gene microarray analysis

To inform this process, I reanalysed unpublished gene microarray (Affymetrix) data generated previously by my supervisor, obtained using cDNAs derived from both bovine and murine parathyroid gland tissues. Tables 3.1 and 3.2 show examples of genes shown to be expressed in those samples. The array data underwent PMA analysis (performed by Leo Zeef, Bioinformatics Core Facility, University of Manchester) with “absent” and “maybe” genes excluded. Then the remaining ~16,000 “probably” expressed genes were ranked according to their chip signal. Although such within-array analysis is semi-quantitative at best, it should be noted
that of the top 10 most apparently abundant parathyroid transcripts, 5 of the bovine and 7 of the murine were ribosomal genes as one would expect (Table 3.1). Similarly, PTH and CaR were highly expressed in both bovine and murine arrays consistent with their crucial role in the regulation of parathyroid gland function (Table 3.1). Furthermore, β-actin and GAPDH were also both in the top 1000. Therefore, there is reason to believe that this crude method of estimating gene expression levels in the PT glands is a useful first step in identifying candidate genes to examine further.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Bovine rank</th>
<th>Murine rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal</td>
<td>5 of top 10</td>
<td>7 of top 10</td>
</tr>
<tr>
<td>PTH</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>CaR</td>
<td>570</td>
<td>2106</td>
</tr>
<tr>
<td>GAPDH</td>
<td>464</td>
<td>707</td>
</tr>
<tr>
<td>β-actin</td>
<td>80</td>
<td>358</td>
</tr>
</tbody>
</table>

Table 3.1. Validation of bovine and murine gene microarrays

Ranking shown out of ~16,000 expressed genes. The cDNAs were obtained from 2 separate bovine PT glands and from one combined sample of multiple mouse PT glands (~ n = 20). The bovine ranking is based on the average of the expression level from the 2 samples.

With this in mind, Table 3.2 then shows some Gαs-coupled receptors and their ligand synthases that were ranked highly in the 16,000 expressed genes in the bovine and murine arrays. These include prostanoid, histamine (in bovine though not murine) and calcitonin-like receptors. Therefore, freshly-isolated bovine parathyroid gland (bPTG) was processed to generate protein particulate fractions and mRNA for subsequent immunoblotting and RT-PCR analyses in order to identify parathyroid-expressed Gαs-coupled receptors.
<table>
<thead>
<tr>
<th>Surface receptor</th>
<th>Gene symbol</th>
<th>Bovine rank</th>
<th>Murine rank</th>
<th>Ligand synthase</th>
<th>Bovine rank</th>
<th>Murine rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandin E receptor 4</td>
<td>EP&lt;sub&gt;4&lt;/sub&gt;</td>
<td>4798</td>
<td>12,421</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>9182</td>
<td>14,849</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PGE synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PGE synthase 3 (cytosolic)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>152</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PG&lt;sub&gt;1&lt;/sub&gt; synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PG&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PG&lt;sub&gt;1&lt;/sub&gt; synthase</td>
<td>11,521</td>
<td>6534</td>
</tr>
<tr>
<td>Prostaglandin I receptor (IP)</td>
<td>IP</td>
<td>N/A</td>
<td>23,816</td>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>9182</td>
<td>14,849</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PGI&lt;sub&gt;2&lt;/sub&gt; synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PG&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PG&lt;sub&gt;1&lt;/sub&gt; synthase</td>
<td>11,521</td>
<td>6534</td>
</tr>
<tr>
<td>Histamine receptor 1</td>
<td>H&lt;sub&gt;1&lt;/sub&gt;</td>
<td>12,216</td>
<td>Absent</td>
<td>Histamine</td>
<td>10,709</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Histidine decarboxylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PG&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PG&lt;sub&gt;1&lt;/sub&gt; synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>11,521</td>
<td>6534</td>
</tr>
<tr>
<td>Surface receptor</td>
<td>Gene symbol</td>
<td>Bovine rank</td>
<td>Murine rank</td>
<td>ligand</td>
<td>Bovine rank</td>
<td>Murine rank</td>
</tr>
<tr>
<td>Calcitonin receptor-like</td>
<td>CALCRL</td>
<td>596</td>
<td>8351</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Receptor activity modifying protein 1</td>
<td>RAMP1</td>
<td>6278</td>
<td>6354</td>
<td>CGRP (α/β)</td>
<td>(β) 13,642</td>
<td>(α) 7966</td>
</tr>
<tr>
<td>+Receptor activity modifying protein 2</td>
<td>RAMP2</td>
<td>2428</td>
<td>2868</td>
<td>Adrenomedullin (AM)</td>
<td>4379</td>
<td>12,449</td>
</tr>
<tr>
<td>+Receptor activity modifying protein 3</td>
<td>RAMP3</td>
<td>4238</td>
<td>17,529</td>
<td>AM &amp; CGRP</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>Adrenergic beta-2 receptor</td>
<td>ADRβ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>6724</td>
<td>12198</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Highly probable expressed genes in bovine and murine microarray analysis.

Ranking shown out of ~16,000 expressed genes. The cDNAs were obtained from 2 separate bovine PT glands and from one combined sample of multiple mouse PT glands (~ n = 20). The bovine ranking based on the average of the expression level from the 2 samples. The receptors and their ligand synthases are shaded with the same colours.
3.3.2 EP₄-receptor expression in bovine parathyroid gland

As stated earlier, preliminary microarray analysis performed here suggested the possible expression of prostanoid receptors including EP₄R and IPR in bovine PT (Table 3.2). Furthermore, there is evidence from the laboratory of our collaborator Prof Arthur Conigrave (Sydney) that selective antagonists of prostanoid receptors and inhibitors of COX-2 can decrease PTH secretion from isolated human parathyroid cells (Szczawinska et al., 2015). Therefore, western blot analysis was carried out to confirm the presence of EP₄R in bovine parathyroid membrane lysate. Bovine PTG particulate fractions (prepared as described in section 2.2) were processed for western blot analysis using anti-EP₄R rabbit polyclonal antibody, resulting in the detection of an EP₄R immunoreactive protein at ~70 kDa. Then, in order to confirm that this band is likely to be EP₄R, deglycosylation of the sample was performed using the enzyme PNGase. In PNGase-treated samples, a smaller, 53 kDa EP₄R-reactive band was detected instead and this corresponds to the expected size of EP₄R (Figure 3.1A). This finding is characteristic of the presence of EP₄R in the bovine parathyroid particulate fraction.

Next, in order to confirm the gene expression of EP₄R in bovine PTG, RT-PCR was performed on the bovine PTG mRNA prepared freshly from PT glands using a Qiagen kit (RNeasy mini kit). Bovine EP₄R gene-specific primers were used to amplify the cDNA made by reverse transcriptions of the total mRNA (prepared as described in section 2.11). By excluding the reverse transcriptase AMV from one reaction (RT-minus) it was confirmed that the DNA amplification detected was from the mRNA as opposed to any genomic DNA contamination. In the agarose gel, EP₄R was observed at 111bp which is identical to the predicted size confirming the gene expression of the EP₄R in bovine PTG (Figure 3.1B).
Figure 3.1. EP4 receptor expression in bovine parathyroid gland.

Panel A) Western blot analysis against EP₄R rabbit polyclonal antibody (1:2000) on bovine PT particulate fractions. PNGase +/- indicates deglycosylation/glycosylation status. Panel B) Representative RT-PCR gel showing EP₄R (111 bp) expression in a bovine PT total mRNA sample. RT +/- indicates the presence or absence of reverse transcriptase (RT) from cDNA-producing step.
3.3.3 EP<sub>4</sub>-receptor transfection in CaR-HEK cells

Transient transfection of prostanoid receptors into CaR-HEK cells was performed to allow investigation of the functional consequences of co-expressing prostanoid receptors together with the CaR. Previous work in this laboratory has shown that raising cAMP levels in CaR-HEK cells using forskolin (an adenylate cyclase activator) enhances the potency of Ca<sup>2+</sup> on the CaR, that is, by lowering the EC<sub>50</sub> for Ca<sup>2+</sup>-induced Ca<sup>2+</sup> mobilisation (Campion, 2013). Therefore, the objective was to see whether co-transfection with either EP<sub>4</sub>R or IPR (both Gα<sub>S</sub>-linked receptors) enhances CaR-induced Ca<sup>2+</sup> mobilisation. Thus, EP<sub>4</sub>R and IPR vectors were transfected into separate CaR-HEK cells using fugene-6 (as described in section 2.7). Control cells were exposed to the same fugene-6 protocol but in the absence of either vector, then western blot analysis was carried out to confirm the success of the transfection process. Anti-EP<sub>4</sub>R rabbit polyclonal antibody was used in immunoblotting against the lysates from control CaR-HEK cells and from cells co-transfected with either IPR or EP<sub>4</sub>R. In the latter case, the EP<sub>4</sub>R lysates were either boiled or non-boiled prior to immunoblotting. An EP<sub>4</sub>R-reactive protein corresponding to the size of EP<sub>4</sub>R was detected in the EP<sub>4</sub>R-transfected cell lysates, whereas only very weak signals were detected in the control and IPR-transfected cells (Figure 3.2A). In the EP<sub>4</sub>R lanes, the signal was much stronger in the non-boiled sample. Together, this confirmed the successful transfection of CaR-HEK cells with EP<sub>4</sub>R and that CaR-HEK cells express EP<sub>4</sub>R endogenously.

Next, to confirm the expression of EP<sub>4</sub>R in the plasma membrane of CaR-HEK cells by immunofluorescence, control (non-transfected) and EP<sub>4</sub>R co-transfected CaR-HEK cells were stained with the anti-EP<sub>4</sub>R primary rabbit polyclonal antibody (1:200) and processed for immunofluorescence. Localisation of immunofluorescent EP<sub>4</sub>R was seen in control cells and the same expression pattern was observed in EP<sub>4</sub>R co-transfected CaR-HEK cells, however it was absent in the negative control sample stained with secondary Alexa Fluor 488 goat anti-rabbit (1:1000) only (Figure 3.2B). This confirmed that this antibody correctly labelled the EP<sub>4</sub>R.
Figure 3.2. Expression of EP₄R in CaR-HEK cells.

3.3.4 PGE synthase 1 and 2 expression in bovine parathyroid gland

After confirming the expression of EP₄R in bovine PT gland, it was necessary to confirm the expression of its ligand synthases in order to support the idea that the PT gland may produce some local factors that could contribute to the intrinsic stimulation of PTH secretion. The cDNA obtained using bovine PT total mRNA, was amplified by RT-PCR using specific primers for PGE synthases 1 and 2. In SafeView-stained agarose gels, 2 bands were observed at the predicted sizes, 203 and 181bp that corresponded respectively to PGE synthase 1 and PGE synthase 2 (Figure 3.3). By eliminating the reverse transcriptase AMV enzyme step from one reaction (RT-), it was confirmed that the amplification products were from total mRNA and not from other genomic DNA contamination.

![Figure 3.3. PGE synthases 1 and 2 expression in bovine parathyroid gland.](image)

Representative RT-PCR gel showing PGE synthase-1 (203 bp) and PGE synthase-2 (181 bp) in a bovine PT total mRNA samples. RT +/- indicates the presence or absence of reverse transcriptase (RT) from cDNA-producing step.
3.3.5 PGE₂ enhances CaR-induced Ca^{2+}_i mobilisation at subthreshold Ca^{2+}_o concentration in CaR-HEK cells

Another key aim of this project was to investigate crosstalk between the CaR and several Gαs-coupled GPCRs. Thus, having confirmed EP₄R expression in bPTG (and in HEK-293 cells), it was then necessary to examine the effect of its ligand PGE₂ on CaR activation, as determined by changes in Ca^{2+}_i mobilisation. In this experiment, HEK-293 cells stably transfected with CaR were used as a model to study CaR signaling. Therefore, CaR-HEK cells loaded with Fura-2 were first incubated in 0.5 mM Ca^{2+}_o-containing buffer and then exposed to a subthreshold Ca^{2+}_o concentration (1.8 mM) in the presence or absence of PGE₂ (100 nM). In this “subthreshold” Ca^{2+}_o concentration, CaR was active enough to induce actin polymerisation (Davies et al., 2006) but not to start Ca^{2+}_i mobilisation in 95% of the cells. From the representative trace in figure (3.4A), it is clear that PGE₂ markedly increased Ca^{2+}_i mobilisation in the presence of a subthreshold Ca^{2+}_o concentration. Quantitative analysis of the area-under-the-curve of the global cell responses confirmed that PGE₂ significantly enhanced Ca^{2+}_i mobilisation at an otherwise subthreshold Ca^{2+}_o concentration (**P<0.01 by Wilcoxon test; n=8) (Figure 3.4B). This effect of PGE₂ will be studied extensively in the next chapter.
Figure 3.4. Effect of PGE on CaR-induced Ca\(^{2+}\) mobilisation at subthreshold Ca\(^{2+}\)\(_{o}\) concentration in CaR-HEK cells.

Panel A) Representative trace showing Ca\(^{2+}\) changes (Fura-2 ratio) in 2 single cells (orange, blue) or ”global” cluster of cells (black) exposed to 1.8 mM Ca\(^{2+}\)\(_{o}\) in presence or absence of PGE\(_2\) (100 nM). Panel B) Quantification of changes in Ca\(^{2+}\) mobilisation as area under the curve/min. Data was normalised to the maximal response and presented as mean ± sem. **P<0.01 by Wilcoxon test; n=8 from 3 independent experiments.
3.3.6 CALCRL expression in bovine PT gland tissue

Next, preliminary data from microarray analysis of cDNA obtained from bovine and murine PTG (Table 3.2) has identified the different components of the CGRP and adrenomedullin (AM) 1 and 2 receptors among the 16,000 likely-to-be-expressed genes. Also, data from our collaborator Professor Conigrave’s laboratory (unpublished data) showed that an AM1 receptor-selective inhibitor leads to increased PTH secretion in PT human cells. Since one of the research objectives was to confirm the expression of Ga<sub>s</sub>-coupled GPCRs involved in the regulation of PTH secretion then, next, western blot and RT-PCR were performed to confirm the expression of CALCRL in bovine PT membrane lysate and total mRNA respectively.

Bovine parathyroid particulate fractions were run for western blot analysis using anti-CALCRL rabbit polyclonal antibody. CALCRL protein expression was confirmed after the detection of the immunoreactive band at ~60 kDa corresponding to the predicted molecular weight of CALCRL protein (Figure 3.5A). In order to confirm the gene expression of CALCRL in bovine PT gland, RT-PCR was performed using CALCRL gene-specific primers to amplify the cDNA obtained from total mRNA by using the reverse transcriptase enzyme (as described in section 2.11). In the resulting agarose gel, a band consistent in size with the 282 bp predicted band was detected in RT+ but not in RT- samples, confirming that this band is most likely produced from gene transcription and not from any other genomic DNA contamination (Figure 3.5B).
Figure 3.5. CALCRL receptor expression in bovine parathyroid gland.

Panel A) Western blot analysis against CALCRL rabbit polyclonal antibody (1:5000) on bovine parathyroid particulate fractions from 2 different glands. Panel B) Representative RT-PCR gel showing CALCRL (282 bp) expression in a bovine PT total mRNA sample. RT +/- indicates the presence or absence of reverse transcriptase (RT) from cDNA producing step.

3.3.7 Other potential cAMP modulating protein expressed in bovine parathyroid gland

Having established the expression of CALCRL in bovine PTG, it was necessary to identify the expression of the other components which associate with CALCRL to form the CGRP and AM 1 and 2 receptors. Again, the previously-mentioned microarray analysis indicated the presence of RAMP-1, RAMP-2 and RAMP-3 mRNA and whose gene products associate with CALCRL to form either CGRP, AM1 or AM2 receptors respectively. Therefore, to confirm the expression of RAMP-1, RAMP-2, RAMP-3 and adrenomedullin expression in the total mRNA of bovine parathyroid gland, RT+/RT- PCRs were carried out using specific primers for each one. In different agarose gels, bands of the appropriate predicted sizes (163, 223, 216, and 202 bp) were detected for RAMP-1, RAMP-2, RAMP-3 and
adrenomedullin receptors respectively (Figure 3.6). Absence of these bands in RT-samples specify that they were produced from gene transcription not from genomic DNA contamination.

**Figure 3.6. CGRP and adrenomedullin receptor forming components expressed in bovine parathyroid total mRNA.**

Representative RT-PCR gels showing RAMP-1 (163 bp), RAMP-2 (223 bp), RAMP-3 (216 bp) and adrenomedullin (202 bp) expression in a bovine PT total mRNA samples. RT +/- indicates the presence or absence of reverse transcriptase (RT) from cDNA producing step.
3.3.8 CALCRL transfection in CaR-HEK cells

In order to achieve the best condition for CALCRL transfection in CaR-HEK cells, I tested different fugene-6 concentrations and different transfection duration. The CaR-HEK cells were transfected with transfection mixture containing 1 µg of vector plus either 3, 4 or 6 µl of fugene-6 and serum free media to make up the final volume to 100 µl. Then the CaR-HEK cells were incubated with the transfection mixture for either 48 or 72 hours prior to western blot analysis using CALCRL primary antibody. The control cells were exposed to the same fugene-6 protocol but in the absence of the CALCRL vector. From the representative western blot, a CALCRL-reactive protein corresponding to the size of CALCRL were detected in all conditions in both control and transfected cells (Figure 3.7). However, in the 72 hours representative immunoblot the signals were much stronger, and this might be due to increased cell density after the third day of incubation. This result confirmed that CaR-HEK cells express CALCRL endogenously and accordingly the non-transfected CaR-HEK cells were used to examine the CGRP effect in the next few experiments.

**Figure 3.7. Expression of CALCRL in CaR-HEK cells.**

Representative western blot showing CaR-HEK cells transfected with 3, 4 or 6 µl fugene-6 in the presence (+CALCRL) or absence (control) of vector. Cells were incubated for 48 or 72 hours with transfection mixture and then blotted against CALCRL antibody.
3.3.9 Effect of CGRP addition during CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation

It has been reported that the CGRP peptide causes activation of different signalling pathways including adenylyl cyclase (AC), PLC and increased Ca\textsuperscript{2+}\textsubscript{i} mobilisation in different cell types (Drissi et al., 1998). Moreover, I have confirmed the expression of CALCRL in bovine PTG both by western blotting and RT-PCR (Figure 3.5) and I have confirmed the gene expression of the other components of CGRP and adrenomedullin receptors in bovine PTG by RT-PCR (Figure 3.6). Consequently, in this experiment the effect of the CGRP on CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation in CaR-HEK cells was examined. Fura-2 loaded cells were first exposed to buffer containing 0.5 mM Ca\textsuperscript{2+}\textsubscript{o}, then to buffer containing 2.5 mM Ca\textsuperscript{2+}\textsubscript{o} to induce Ca\textsuperscript{2+}\textsubscript{i} mobilisation. Next, CGRP (2 nM) was further added to the buffer. From the representative trace in Figure (3.8A), further treatment with CGRP caused a significant increase in Ca\textsuperscript{2+}\textsubscript{i} mobilisation, seen as an increase in the amplitude and frequency of the oscillations. Quantification of the AUC of the global cell responses confirmed that CGRP had significantly increased the CaR-induced Ca\textsuperscript{2+}\textsubscript{i} at 2.5 mM Ca\textsuperscript{2+}\textsubscript{o} concentration by Wilcoxon test; n=23 (Figure 3.8B).
Figure 3.8. Effect of CGRP in 2.5 mM Ca\textsuperscript{2+},induced Ca\textsuperscript{2+}i mobilisation.

Panel A) Representative trace showing Ca\textsuperscript{2+}, changes (Fura-2 ratio) in 2 single cells (orange, blue) or "global" cluster of cells (black) exposed to 2.5 mM Ca\textsuperscript{2+}, in presence or absence of CGRP (2 nM). Panel B) Quantification of changes in Ca\textsuperscript{2+}, mobilisation as area under the curve/min. Data was normalised to the maximal response and presented as mean ± sem. **P<0.01 by Wilcoxon test; n=23 from 6 independent experiments.
3.3.10 Effect of CGRP on CaR-induced Ca$^{2+}_i$ mobilisation as a function of extracellular calcium concentration

Next, to determine whether CGRP increases CaR sensitivity to Ca$^{2+}_o$ concentration, intracellular calcium imaging was performed on CaR-HEK cells exposed to increasing Ca$^{2+}_o$ concentrations (0.5, 2, 3, 5 and 10 mM) in the presence or absence of 2 nM CGRP. From the representative traces, CGRP did not significantly enhance CaR-induced Ca$^{2+}_i$ mobilisation in comparison with control cells (Figure 3.9A). From the sigmoidal concentration-effect curves, the EC$_{50}$ value (3.9 ± 0.4) for Ca$^{2+}_o$ in CGRP co-treated CaR-HEK cells was, if anything, slightly higher than the EC$_{50}$ (3.6 ± 0.2) for the control group, but they were not significantly different when analysed using the Mann-Whitney test (two-tailed); n=8 (Figure 3.9B). The Hill coefficient for the sigmoidal dose response curves fitted as described in (section 2.14) are 3.9 for control and 3.4 for CGRP (Figure 3.9B).
Figure 3.9. Effect of CGRP on Ca\textsuperscript{2+}\textsubscript{o}–induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation in CaR-HEK cells.

Panel A) Representative traces showing Ca\textsuperscript{2+}\textsubscript{i} changes (Fura-2 ratio) in 2 single cells (orange, blue) or "global" cluster of cells (black) in response to increasing Ca\textsuperscript{2+}\textsubscript{o} concentrations (0.5-10 mM) in CaR-HEK cells (i) or plus CGRP (2 nM) (ii). Panel B) Quantification of the responses are shown as sigmoidal dose response curves. Data was normalised to the maximal response. (ns) not-significant vs. control EC\textsubscript{50} by Mann-Whitney test (two-tailed); n=8 from 3 independent experiments.
3.3.11 Secondary anti-goat antibody cross-react with bovine parathyroid particulate fractions

Next, I attempted to investigate the expression of the prostanoid receptor (IPR) and histamine 2 receptor (H₂R) in bovine PT membrane lysate by western blotting. For this, two primary antibodies raised in goat were employed to detect IPR (N-20) and (Q-13) while another goat antibody was used to detect H₂R (A-20). All 3 antibodies produced substantial immunoreactivity of approximate molecular mass consistent with the bands been bovine parathyroid IPR and H₂R proteins. However, cotreatment of these antibodies with their immunising peptides failed to ablate the resulting signal (Figure 3.10A/B). Similarly, preincubation of the parathyroid particulate lysates with PNGaseF failed to lower the apparent molecular mass of these proteins which might have been expected for membrane proteins which are invariably glycoproteins (Figure 3.10A/B). Therefore next, the westerns were probed only with either the donkey anti-goat IgG (sc-2020) or rabbit anti-goat secondary IgG (sc-2922) and again the same highly abundant bands were detected (Figure 3.10C) suggesting that the bands are most likely non-specific.

Thus, further bovine parathyroid particulate samples were then resolved using an 8% SDS-PAGE gel with the gel then stained with Coomassie blue with shaking (15 minutes) followed by destaining overnight. A section of the gel consistent with the size of the highly immunoreactive band was removed and sent for mass spectrometry analysis. The two most highly abundant proteins in the band were albumin and chromogranin. In order to determine whether it was albumin that was the immunoreactive, albumin was run beside the bovine PT membrane lysate in a western blot and then probed with the secondary anti-goat. However, in the lane where the albumin was run, there was no immunoreactive signal detected (Figure 3.10D). Based on these findings therefore, the secondary anti-goat appears to be cross-reacting with an endogenous protein or possibly an immunoglobulin present in bovine PT membrane lysate. But in any case the signal appeared ultimately artefactual and thus was not examined further.
Figure 3.10. Secondary anti-goat antibody cross-react with bovine parathyroid particulate fractions.

Panel A) and B) Western blot analysis against IPR and H2R goat polyclonal antibody (1:5000) respectively on bovine PT particulate fractions. PNGase +/- indicates deglycosylation/glycosylation status. Peptide +/- indicates presence or absence of the specific receptor-antibody peptide. Panel C) Western blot analysis against secondary donkey anti-goat (left lane) and rabbit anti-goat (right lane) on bovine PT particulate fractions. Panel D) Western blot analysis against secondary anti-goat on bovine PT particulate fractions (left lane) and albumin (right lane).

3.3.12 Identification of certain Gaα-coupled GPCRs in murine PT gland preincubated in high, normal or low Ca\textsuperscript{2+} as revealed by gene microarray analysis

Finally, following the initial microarray analysis of bovine and murine PT mRNA samples performed in conjunction with Professors Arthur Conigrave (Sydney) and Wenhan Chang (UCSF) to identify candidate Gaα-coupled GPCRs and their ligand synthases (Table 3.1 & 3.2), next we examined whether altering Ca\textsuperscript{2+} concentrations may affect the expression of any of our genes of interest. Specifically, Prof Chang incubated murine PTs for 6 hours in high (1.6 mM), normal (1.3 mM) or low (1.0 mM) Ca\textsuperscript{2+} prior to mRNA extraction. Then the same were analysed by Affymetrix microarray here in Manchester with ~20,000 genes predicted to be expressed. I then
re-interrogated the array data to assess whether expression any of the proteins detailed in this chapter may be regulated by $\text{Ca}^{2+}_o$.

As explained earlier, this microarray analysis is only semi-quantitative at best, however 13 of the 20 most abundant genes were ribosomal which is what would be expected. More importantly though is that the calciotropic genes PTH, CaR and VDR all exhibited linear changes between high, normal and low $\text{Ca}^{2+}_o$ concentrations in the directions expected. That is, PTH was downregulated with high $\text{Ca}^{2+}_o$ (hypercalcaemia) and up-regulated with low $\text{Ca}^{2+}_o$ (Table 3.3) which would occur in vivo to facilitate a rise in serum $\text{Ca}^{2+}_o$ concentration back to normal. Next, CaR gene was up-regulated in the presence of high $\text{Ca}^{2+}_o$ concentration but nearly absent with low $\text{Ca}^{2+}_o$ concentration (Table 3.3) in line with CaR’s ability to suppress PTH i.e. lowering CaR expression will mean more PTH secretion whereas raising CaR expression will suppress PTH secretion. Finally, VDR expression changed in the same direction as CaR (Table 3.3) which would make sense as it also acts to suppress PTH expression and secretion. Together these findings validate the gene arrays as physiologically-relevant indicators of calciotropic gene expression. Thus, next I examined the effect of altering $\text{Ca}^{2+}_o$ concentrations on the expression of $\text{G} \alpha_s$-linked GPCRs as well as of their ligand synthases. As can be seen in Table 3.3, the only other “calciotropic” genes appear to be $\text{Ca}^{2+}_o$-sensitive was the Calcitonin receptor-like receptor (CALCRL) as well as the genes for its ligands, calcitonin-related polypeptides $\alpha$ and $\beta$. Since these changes were in the same direction as for CaR and VDR then one might speculate that they also suppress PTH secretion however this would need to be specifically tested.

However, of the other potentially calciotropic genes examined in this chapter, none exhibited changes that were linear with respect to $\text{Ca}^{2+}_o$ concentrations i.e. either 1.6 > 1.3 > 1.0 or 1.6 < 1.3 < 1.0. Therefore, even if the prostanoid receptors do drive PTH secretion, their expression is not apparently regulated in a feedback manner by the resulting change in $\text{Ca}^{2+}_o$. 

81
<table>
<thead>
<tr>
<th>Surface receptor</th>
<th>Gene symbol</th>
<th>Rank</th>
<th>Signals in high Ca&lt;sup&gt;2+&lt;/sup&gt; vs. normal Ca&lt;sup&gt;2+&lt;/sup&gt; (%)</th>
<th>Signals in low Ca&lt;sup&gt;2+&lt;/sup&gt; vs. normal Ca&lt;sup&gt;2+&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parathyroid hormone</td>
<td>PTH</td>
<td>16</td>
<td>-30</td>
<td>24</td>
</tr>
<tr>
<td>Calcium-sensing receptor</td>
<td>CaSR</td>
<td>16366</td>
<td>93</td>
<td>-63</td>
</tr>
<tr>
<td>Vitamin D receptor</td>
<td>VDR</td>
<td>2256</td>
<td>36</td>
<td>-34</td>
</tr>
<tr>
<td>Calcitonin receptor-like</td>
<td>CALCRL</td>
<td>8927</td>
<td>15</td>
<td>-10</td>
</tr>
<tr>
<td>Receptor activity modifying protein 1</td>
<td>Ramp1</td>
<td>18818</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receptor activity modifying protein 2</td>
<td>Ramp2</td>
<td>18672</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receptor activity modifying protein 3</td>
<td>Ramp3</td>
<td>14865</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenomedullin</td>
<td>ADM</td>
<td>5511</td>
<td>75</td>
<td>18</td>
</tr>
<tr>
<td>calcitonin-related polypeptide, alpha</td>
<td>CGRP(α)</td>
<td>14330</td>
<td></td>
<td></td>
</tr>
<tr>
<td>calcitonin-related polypeptide, beta</td>
<td>CGRP(β)</td>
<td>25016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>prostaglandin E receptor 4</td>
<td>EP4</td>
<td>12593</td>
<td>0</td>
<td>-17</td>
</tr>
<tr>
<td>prostaglandin E synthase</td>
<td>Ptges</td>
<td>9792</td>
<td>-8</td>
<td>-22</td>
</tr>
<tr>
<td>prostaglandin E synthase 2</td>
<td>Ptges2</td>
<td>10412</td>
<td>-23</td>
<td>2</td>
</tr>
<tr>
<td>prostaglandin I receptor (IP)</td>
<td>IP</td>
<td>16781</td>
<td>145</td>
<td>72</td>
</tr>
<tr>
<td>prostaglandin I2 (prostacyclin) synthase</td>
<td>Ptgis</td>
<td>6862</td>
<td>42</td>
<td>33</td>
</tr>
<tr>
<td>adrenergic receptor, beta 2</td>
<td>Adrβ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>14147</td>
<td>-23</td>
<td>-43</td>
</tr>
</tbody>
</table>

Table 3.3. Table of selected receptors and their ligand synthases from mouse microarray data.

Ranking based on expression level in normal [Ca<sup>2+</sup>]<sub>o</sub> (1.3 mM). The cDNA obtained from different PT glands treated with either high (1.6 mM), normal (1.3 mM) or low (1 mM) [Ca<sup>2+</sup>]<sub>o</sub>. Red cells indicate downregulation and green cells indicate upregulation but this only shown for genes that were modulated by extracellular Ca<sup>2+</sup> in linear fashion.
3.4 Discussion

3.4.1 Identification of candidate intrinsic regulators of PTH secretion

The negative feedback mechanism that regulates PTH secretion and which involves CaR activation and G\(\alpha_q/11\) stimulation is well appreciated if not particularly well understood mechanistically. However, there is a distinct lack of understanding concerning the driving force eliciting PTH secretion when serum Ca\(^{2+}\) concentrations are low. This is primarily because PTH has no secretion-stimulating signal like for other endocrine hormones such as thyroid hormone and cortisol which have the pituitary-stimulating hormones; TSH and ACTH respectively. There is significant evidence to suggest that increasing intracellular cAMP levels can elicit PTH secretion (Brown et al., 1977a; Brown et al., 1977b; Gardner et al., 1978; Shoback and Brown, 1984) and this would imply that raising cAMP through the activation of G\(\alpha_s\)-coupled GPCRs could underpin such a process, with the next question being which G\(\alpha_s\)-coupled receptors might be involved in this?

3.4.2 EP\(_4\)-receptor expression in bovine parathyroid gland

Having confirmed the protein and gene expression of EP\(_4\)R in bovine PTG tissue, this supports the findings of previous studies suggested that increased cAMP production by prostaglandins such as PGE\(_2\) and thromboxane \(\beta_2\), elicit PTH secretion in dispersed bovine parathyroid cells (Gardner et al., 1978; Gardner et al., 1980; Brown and Swartz, 1985). Moreover, in our lab the expression of some prostaglandin receptors such as (EP\(_4\)R and IPR) and their ligand synthases were indicated in the microarray analysis of cDNA obtained from bovine and murine parathyroid glands (Table 3.2). Interestingly, in the mouse array that tested the effect of 6-hour pretreatment with either 1.0, 1.3 or 1.6 mM Ca\(^{2+}\), the level of expression of EP\(_4\)R and the two PGE synthases were apparently not affected by Ca\(^{2+}\) concentration (Table 3.3) whereas the relative expression of CaR, VDR and PTH were each Ca\(^{2+}\)-sensitive in the manner predicted. This implies that even if EP\(_4\)R receptor is involved in driving PTH secretion, there is unlikely to be genomic feedback from the CaR to regulate this. Although to prove this conclusively, more quantitative analyses with sufficient statistical power will be required.
Significantly, our collaborators have shown that cotreatment with the COX2 inhibitor NS398 rapidly and reversibly halves PTH secretion in perifused human parathyroid cells, where \( \text{Ca}^{2+} \) concentration is low (1.0 mM). In addition, both the broad-spectrum prostanoid receptor inhibitor AH6809 as well as the selective inhibitors of the EP\(_4\) receptor L-161,982 and prostacyclin IP1 receptor Ro1138452 both inhibited PTH secretion in a rapid and reversible manner in the same model (Szczawinska et al., 2015).

Thus, the apparent expression of the \( G_\alpha_s \)-coupled GPCR EP\(_4\)R in both bovine and murine parathyroid gland together with the fact that pharmacological inhibition of EP\(_4\)R suppresses PTH secretion supports the idea that the parathyroid gland maintains spontaneous release of PTH through an intrinsic mechanism.

3.4.3 EP\(_4\)-receptor transfection in CaR-HEK cells

In the continuing absence of a suitable parathyroid cell line model and the difficulty of obtaining sufficient recovery of primary bovine PT cells following collagenase digestion, the next best way to examine possible crosstalk between CaR and \( G_\alpha_s \)-coupled GPCRs are using CaR-transfected HEK-293 cells. It is worth noting that while clearly different from PT cells, CaR-HEK cells do still exhibit similar signalling responses to \( \text{Ca}^{2+} \) to parathyroid cells namely, IP\(_3\)-induced \( \text{Ca}^{2+} \) mobilisation (including oscillations), decreased cAMP generation and MAP kinase activation and neither cell-type are electrically excitable (McCormick et al., 2010).

As mentioned earlier, microarray analysis of murine parathyroid glands treated with high, low and normal \( \text{Ca}^{2+} \) (Table 3.3) suggests that EP\(_4\)R expression does not follow linear \( \text{Ca}^{2+} \)-dependency suggesting that the abundance of the prostanoid receptor is not obviously \( \text{Ca}^{2+} \) sensitive. If true, and this would require more quantitative testing to confirm, then it would be more likely that the prostanoid receptor may regulates PTH secretion via signalling crosstalk between the prostanoid receptors and CaR. Thus, by confirming that the CaR-HEK cells were successfully transfected with EP\(_4\)R but also that they express some EP\(_4\)R endogenously, this renders the CaR-HEK cells as a reasonable model to use in the signalling crosstalk experiments in the next chapter.
It has been reported that many GPCRs undergo internalisation upon activation, meaning translocation of the receptor from the cell membrane to intracellular vesicles (Koenig and Edwardson, 1997; Desai and Ashby, 2001). Indeed, Desai et al. (2000) found that EP₄R internalised rapidly upon PGE₂ stimulation whereas EP₂R did not internalise. However, there is little known about whether or not EP₄R could internalise upon activation of another receptor. As my aim is to study the crosstalk between the Gαₛ-coupled and Gα₉/₁₁-coupled GPCRs, I attempted to see if CaR activation induced EP₄R internalisation using an immunofluorescence technique. The EP₄R immunoreactivity seen in the cells was punctate with little evidence of strong membrane localisation and the endogenous EP₄R expression was sufficient to give robust immunofluorescence, which was heightened a little by EP₄R transfection. Significantly, the localisation of EP₄R in control non-transfected cells was very similar in appearance to that found in EP₄R-cotransfected CaR-HEK cells. That is, there was no gross change in the brightness of the signal on the membrane relative to its brightness in the cytosol. This result appears consistent with a study investigating the effect of PGE₂ in the production of amyloid-β peptide through internalisation of EP₄R. HEK-293 cells were used without need of EP₄R co-transfection, suggesting that HEK-293 cells express EP₄R endogenously (Hoshino et al., 2009). In the absence of sufficient discrimination between membrane and intracellular localisation, this technique was not used further or developed to be more quantitative.

3.4.4 PGE synthase 1 and 2 expression in bovine parathyroid gland

PGE synthases produce PGE₂ from prostaglandin H₂; a product of arachidonic acid by the action of COX enzyme. There are three known isomerases of PGE synthases; two microsomal (PGE synthase 1 and 2) and one cytosolic (PGE synthase 3). The microsomal mPGES-1 selective inhibitor inhibits the formation of PGE₂ but not other prostaglandins including PGI₂ and TXA₂ (Kawabata, 2011). As previously mentioned, it was reported in 1985 that prostaglandins such as PGE₂ and TXA₂ cause PTH release from dispersed bPT cells by generating cAMP (Gardner et al., 1978; Gardner et al., 1980; Brown and Swartz, 1985). However, there are no studies linking these findings with the intrinsic or tonic secretion of PTH. Moreover, the confirmed gene expression of PGE synthases 1/2 is consistent with the bovine and
murine genes arrays data that detected PGE synthases 1 and 2 among the top 16,000 likely-to-be-expressed genes (Table 3.2). Furthermore, unpublished findings from our collaborator Professor Conigrave, suggest that inhibition of prostaglandin synthesis with a COX inhibitor decreases PTH secretion in a dose-dependent manner in human PTG cells exposed to low (1 mM) Ca\(^{2+}\) (Szczawinska et al., 2015). These findings support the idea that the PTG produces factors locally, which may activate \(G_\alpha\)-linked GPCRs in a paracrine/autocrine fashion to increase cAMP generation and thus stimulate PTH secretion (Conigrave, 2016).

### 3.4.5 PGE\(_2\) enhances CaR-induced Ca\(^{2+}\)\(_i\) mobilisation in CaR-HEK cells at subthreshold Ca\(^{2+}\)\(_o\) concentration

PGE\(_2\) stimulates EP\(_4\)R causing activation of adenylate cyclase and thus increasing cAMP level inside the cells (Desai et al., 2000; Li et al., 2017) and a number of studies indicate that in PTG cells, increased cAMP generation causes PTH secretion (Brown et al., 1977a; Brown et al., 1977b; Gardner et al., 1978; Shoback and Brown, 1984) reviewed in (Brown and MacLeod, 2001). In contrast, CaR-induced Ca\(^{2+}\)\(_i\) mobilisation is known to have a powerful cAMP-inhibitory action, either through activation of the \(G_\alpha\) intracellular signaling pathway, or, activation of a phosphodiesterase (PDE) (Houslay and Milligan, 1997; Goraya et al., 2004; Conigrave and Ward, 2013). According to Gerbino et al. (2005), increased cAMP generation in CaR-HEK cells using PGE\(_2\), enhanced the frequency and the amplitude of spermine-induced Ca\(^{2+}\)\(_i\) oscillations. This increase in Ca\(^{2+}\)\(_i\) mobilisation due to more cAMP generation provides an additional inhibitory effect of CaR-induced cAMP suppression. In vivo, the benefit of having such an inhibitory (“short-loop feedback”) effect would be that PTH secretion could be reduced even prior to Ca\(^{2+}\)\(_o\) having risen enough to elicit CaR-mediated PTH suppression (“long-loop feedback”). Here my result confirmed that PGE\(_2\) enhanced CaR-induced Ca\(^{2+}\)\(_i\) mobilisation even in a Ca\(^{2+}\)\(_o\) concentration that does not initiate \(G_\alpha_{q/11}\)-induced Ca\(^{2+}\)\(_i\) mobilisation in CaR-HEK cells (Davies et al., 2006). My result is also consistent with another study conducted previously in our lab which showed that forskolin (cAMP-elevating compound) enhanced CaR-induced Ca\(^{2+}\)\(_i\) mobilisation at sub-threshold Ca\(^{2+}\)\(_o\) concentrations (Campion, 2013). This finding forms the basis of a more in-depth investigation of this apparent crosstalk between cAMP and CaR
signalling in the next chapter.

3.4.6 CALCRL and other potential cAMP-generating receptors in bovine PT gland

The CALCRL makes CGRP, AM1 or AM2 when it combines with RAMP-1, RAMP-2 or RAMP-3 respectively (Conner et al., 2005). Confirming the protein and gene expression of CALCRL in bovine PTG tissue support the results of microarray analysis mentioned in (Table 3.2). Additionally, all known RAMPs that act as chaperones to enhance the cell surface expression of CALCRL were indicated in the top 16,000 likely to be expressed genes in both bovine and murine microarrays and my result has confirmed this. The importance of RAMPs in PTG could be explained by what has been reported in much of the literature; that RAMP-1 and RAMP-3 but not RAMP-2 also act as chaperones to introduce the class C GPCR (CaR) to the cell surface (Bouschet et al., 2005; Desai et al., 2014; Hay and Pioszak, 2016) This was found when RAMP-1 knockdown caused inhibition of cinacalcet-induced Ca\textsuperscript{2+} mobilisation response in medullary thyroid carcinoma TT cells (Desai et al., 2014). Furthermore, in the mouse gene array comparing the effect of Ca\textsuperscript{2+} concentrations (Table 3.3) the CALCRL gene as well as those for its ligand CGRP α and β were upregulated in the gland incubated in high Ca\textsuperscript{2+} concentrations and down-regulated in low Ca\textsuperscript{2+} concentrations suggesting that the genes of this system may be influenced by Ca\textsuperscript{2+} similarly to the other known calciotropic genes; CaR, PTH and VDR. That said, the relative expression levels of the RAMPs were not affected by Ca\textsuperscript{2+} (Table 3.3).

3.4.7 CALCRL transfection in CaR-HEK cells

Next, to study the cross-talk between CaR and CALCRL, CaR-HEK cells were transiently transfected with CALCRL vector (Figure 3.7). My findings, however, suggested that CaR-HEK cells expressed CALCRL endogenously as a single immunoreactive band of the appropriate size for CALCRL was detected in both control and transfected cells and that CALCRL transfection did not appear to enhance the level of expression. This finding is contrary to a study which found that CGRP failed to increase cAMP level in HEK-293 cells, suggesting that HEK-293 cells lack the functional component of the CGRP receptor (Atwood et al., 2011). In
addition, Terra *et al.* (2015), found that hCGRP was able to induce cAMP in CALCRL/RAMP-1 co-transfected HEK-293 cells and that the CGRP needs the CALCRL/RAMP-1 complex to function. However, it has been reported by Hay and Pioszak that RAMP-1 but not RAMP-2 and 3 is endogenously expressed in HEK-293 cells (Hay and Pioszak, 2016). In concordance with my finding, non-transfected CaR-HEK cells were used in the ensuing experiments to study the signalling crosstalk between CGRP and CaR.

### 3.4.8 Effect of CGRP addition during CaR-induced $\mathrm{Ca}^{2+}_i$ mobilisation

Class B GPCRs, including CALCRL, are commonly coupled to the $\mathrm{G}_{\alpha_s}$ intracellular signalling pathway (Terra *et al.*, 2015; Weston *et al.*, 2016; Woolley *et al.*, 2017). By stimulating with CGRP, the CALCRL/RAMP-1 complex is being activated as CGRP is known to activate CGRP receptor with high affinity (McLatchie *et al.*, 1998; Woolley *et al.*, 2017). In 1996, Aiyar *et al.* found that in HEK-293 cells co-transfected with CALCRL, CGRP caused a 60-fold increase in cAMP levels (Aiyar *et al.*, 1996). In contrast, another article reported that CGRP receptor could mediate different intracellular signalling pathways including $\mathrm{G}_{\alpha_s}$, $\mathrm{G}_{\alpha_q/11}$, $\mathrm{G}_i$, and $\beta$-arrestin (Russell *et al.*, 2014). Moreover, it has been reported that CGRP activates an adenylate cyclase enzyme to increase cAMP level in osteoblasts (Naot and Cornish, 2008), however in human bone cells (OHS-4) it only activates the PLC pathway (Drissi *et al.*, 1999). It has also been reported by Drissi *et al.* (1998), that CGRP could stimulate dual signalling through the activation of both $\mathrm{G}_{\alpha_s}$ and $\mathrm{G}_{\alpha_q/11}$. This suggests that in the case of CGRP, the cell-type directly influences the G protein signalling pathway. In the current study, it was found that CGRP enhanced CaR-induced $\mathrm{Ca}^{2+}_i$ mobilisation in the presence of a moderate $\mathrm{Ca}^{2+}_o$ concentration (Figure 3.8) though it is unknown by which intracellular mediator this effect occurred i.e. due to increased cAMP production or directly due to elevated $\mathrm{Ca}^{2+}_i$ mobilisation. However, when CGRP was used in longer experiments to determine whether it could significantly reduce the EC$_{50}$ for $\mathrm{Ca}^{2+}_o$-induced $\mathrm{Ca}^{2+}_i$ mobilisation, no such effect was seen (Figure 3.9). This might be explained by findings from Kuwasako *et al.* (2000), who reported that in HEK-293 cells, CALCRL internalised into lysosomes for degradation upon binding of the agonist CGRP. Indeed, internalisation is a classic mechanism for desensitising GPCRs (Mundell and Kelly, 1998; Kliewer *et
Thus, there may be a very brief stimulatory effect of CGRP that is not sufficiently sustained in these cells to perform longer experiments. Thus, while this receptor was not further studied in the project, it may still be significant that CALCRL mRNA and protein expression was observed in the bovine PTG, and that its mRNA expression was apparently Ca$^{2+}$-sensitive in the murine microarrays (Table 3.3).

3.4.9 Secondary anti-goat antibody cross-react with bovine parathyroid particulate fractions

With regards the significant immunoreactive signal seen using the anti-goat secondary, there are no previous reports to my knowledge suggesting that secondary anti-goat antibody cross-reacts with endogenous immunoglobulin(s) or protein(s) in bovine PT gland tissue. Nevertheless, this does appear to be the case since using two different primary antibodies both raised in goat against different proteins, the same highly immunoreactive bands were detected and were similarly detected even when only the secondary anti-goat antibody was employed. This unfortunate artefact hampered progress for some time, as the initial assumption was that the strong signal observed was due to specific binding of the primary antibody. No further experiments were performed with these or other goat-derived primary antibodies.

3.5 Summary

The expression of several $\text{G}_{\alpha}$-coupled GPCRs, their ligand synthases or associated components were confirmed in bPTG tissue. Most notable was $\text{EP}_4\text{R}$ and the PGE synthases as well as CALCRL (and RAMPs 1-3).
CHAPTER 4

Signal Crosstalk between CaR and Other Parathyroid GPCR Signals
4.1 Introduction

Activation of \(\mathrm{G}\alpha_s\)-coupled GPCRs activate the enzyme adenylyl cyclase and thus increase intracellular cAMP levels, while \(\mathrm{G}\alpha_{q/11}\)-coupled GPCRs activate phospholipase C to elicit IP\(_3\) and DAG generation. A great many receptors, expressed together in the same cell, are capable of one or both of these features and yet their functional interactions tend to be under-investigated. That is, most experimental protocols involve removing as many stimuli as possible from the system and then testing the consequence of restoring a very large single stimulus. While reductionism is necessary to examine individual pathways, our physiology *per se* involves networks of concomitant signalling pathways interacting to achieve homeostasis overall. Therefore, it is also necessary to examine how signals interact or crosstalk with one another.

In the previous chapter it was demonstrated that the cAMP generator PGE\(_2\) enhances CaR-induced \(\mathrm{Ca}^{2+}\) mobilisation in subthreshold \(\mathrm{Ca}^{2+}\) concentrations in CaR-HEK cells. In this chapter, I will then investigate the crosstalk between CaR and some \(\mathrm{G}\alpha_s\)-coupled GPCRs as determined by changes in \(\mathrm{Ca}^{2+}\) mobilisation and ERK activation in CaR-HEK cells. The ultimate aim of this work is to model how cAMP generation by one or more \(\mathrm{G}\alpha_s\)-coupled GPCRs may elicit PTH secretion, while the CaR then moderates this effect to limit PTH secretion and thus maintain \(\mathrm{Ca}^{2+}\) homeostasis.

4.2 Materials and Methods

CaR-HEK cells were cultured as described in section 2.6. Transient cell transfection was performed as mentioned in section 2.7. Intracellular calcium imaging (section 2.9), cell assay and lysate preparation (section 2.8), immunoblotting (section 2.3) were performed as described in named sections.
4.3 Results

4.3.1 Effect of EP₄ receptor co-transfection on CaR-induced Ca²⁺ mobilisation in CaR-HEK cells

Having demonstrated EP₄R gene and protein expression in bovine parathyroid gland (Figure 3.1) and shown successful transfection of exogenous EP₄R in CaR-HEK cells (Figure 3.2), it was necessary to examine how the co-expression of this Gα₅-linked GPCR affects the intracellular signalling of the Gαq-coupled CaR in response to increased Ca²⁺o concentrations.

Therefore, in this experiment Fura-2 loaded CaR-HEK cells incubated with transfection buffer in the absence or presence of EP₄R vector were exposed to increasing Ca²⁺o concentrations (0.5, 2, 3, 5 and 10 mM). From the representative traces showing the Ca²⁺o concentration-dependent changes in Ca²⁺i mobilisation (Figure 4.1A), EP₄R ambient co-expression did not enhance CaR-induced Ca²⁺i mobilisation. From the sigmoidal concentration-effect curves in (Figure 4.1B), the EC₅₀ value (defined as the [Ca²⁺]₀ needed to elicit 50% of the maximal CaR response) for Ca²⁺o in EP₄R co-transfected CaR-HEK cells (4.2 ± 0.4) was not statistically different from the EC₅₀ for the control group (4.2 ± 0.2). The Hill coefficient for the sigmoidal dose response curves fitted as described in (section 2.14) are 3.4 for control and 3.0 for EP₄R (Figure 4.1B). This suggests that simple co-expression with EP₄R does not enhance CaR-induced Ca²⁺i mobilisation and that EP₄R is not constitutively active here.
Figure 4.1. Effect of increasing \(Ca^{2+}_o\) concentration on \(Ca^{2+}_i\) mobilisation in CaR-HEK cells co-transfected with EP\(_4\)R.

Panel A) Representative traces showing \(Ca^{2+}_i\) changes (Fura-2 ratio) in 2 single cells (orange, blue) or "global" cluster of cells (black) in response to increasing \(Ca^{2+}_o\) concentrations (0.5-10 mM) in CaR-HEK cells (i) and in CaR-HEK cells co-transfected with EP\(_4\)R (ii). Panel B) Quantification of the responses are shown as sigmoidal dose response curves. Data was normalised to the maximal response. (ns) not-significant vs. control EC\(_{50}\) by Mann Whitney test (two-tailed); \(n=17-18\) from 6 independent experiments.
4.3.2 Effect of Indomethacin on CaR-induced Ca$^{2+}$i mobilisation in CaR-HEK cells co-transfected with EP$_4$R

The prostaglandin production inhibitor indomethacin was used to investigate if its addition might affect the sensitivity of CaR-induced Ca$^{2+}$i mobilisation in CaR-HEK cells co-transfected with EP$_4$R. This was carried out to see whether EP$_4$R is activated as a result of locally-produced prostaglandin release, or, whether they may instead be constitutively active. Therefore, Fura-2 loaded EP$_4$R-transfected CaR-HEK cells were exposed to either 1 or 10 µM indomethacin for 15 minutes in 0.5 mM Ca$^{2+}$o-containing buffer prior to treatment with 2 then 3 mM Ca$^{2+}$o in the continued presence of the indomethacin. Co-treatment with indomethacin at either 1 or 10 µM failed to inhibit the CaR responses to either 2 or 3 mM Ca$^{2+}$o (Figure 4.2A). This involved quantification of the area under the curve (AUC) in response to elevated [Ca$^{2+}$]$_o$ and compared by one-way ANOVA with Dunnett’s multiple comparisons post-test. This revealed that indomethacin had no effect on CaR-induced Ca$^{2+}$i mobilisation in CaR-HEK cells co-transfected with EP$_4$R (Figure 4.2B). If anything, the AUC with both 2 and 3 mM Ca$^{2+}$o appeared higher in 10 µM indomethacin-treated cells but this increase was not significant and may result from a single outlying value.
Figure 4.2. Effect of indomethacin on CaR-induced Ca^{2+}_{i} mobilisation in CaR-HEK cells co-transfected with EP_{4}R.

Panel A) Representative traces showing Ca^{2+}_{i} changes (Fura-2 ratio) in 2 single cells (orange, blue) or“global” cluster of cells (black) when stimulated with 2 and then 3 mM Ca^{2+}_{o} in the absence (i) and then presence of either 1 µM (ii) or 10 µM (iii) indomethacin (in 0.5 mM Ca^{2+}_{o} unless otherwise stated). Panel B) Quantification of these changes shown as area under the curve (AUC %) in response to elevated [Ca^{2+}]_{o}. (ns) not-significant by one-way ANOVA with Dunnett’s multiple comparisons post-test; n=5-9 from 3 independent experiments.
4.3.3 Effect of EP₄R co-transfection in CaR-HEK cells on CaR-induced ERK phosphorylation

It is well established that increasing Ca²⁺ₒ in CaR-HEK cells enhances the phosphorylation of the MAP kinase ERK (Kifor et al., 2001). Thus, having shown that co-expression of EP₄R in CaR-HEK cells does not enhance the sensitivity of CaR-induced Ca²⁺ᵢ mobilisation, I next investigated whether co-expression with EP₄R could modulate CaR-induced ERK phosphorylation or not. Thus, in this experiment, either wild type or EP₄R co-transfected CaR-HEK cells were exposed to increasing Ca²⁺ₒ (0.5, 2, 3 and 5 mM) concentrations (at 37°C for 10 minutes) then lysed and immunoblotted against the p-ERK antibody. The successful transfection of EP₄R was confirmed by increasing EP₄R expression in transfected CaR-HEK cells versus control non-transfected cells by immunoblotting as showed in Figure 3.2A. As shown in Figure 4.3A, raising Ca²⁺ₒ concentrations from 0.5 to 5 markedly increased the phosphorylation level of ERK in both control and EP₄R co-transfected CaR-HEK cells. Data quantification by densitometry demonstrated that the responses to 0.5, 2, 3 or 5 mM Ca²⁺ₒ were not elevated in EP₄R transfected cells (p>0.05) relative to wild-type. That is, between the resulting sigmoidal concentration-effect curves, there was no statistical difference between the EC₅₀ for Ca²⁺ₒ in EP₄R-transfected cells (2.5 ± 0.1 mM) versus control cells (2.5 ± 0.0). The Hill coefficient for the sigmoidal dose response curves fitted as described in (section 2.14) are 6.4 for control and 5.7 for EP₄R (Figure 4.3B). From these data, it appears that EP₄R co-expression has no effect on CaR-induced ERK phosphorylation in CaR-HEK cells or that the receptor requires locally produced prostaglandin to enhance CaR responsiveness. Therefore, in the following experiments, non-transfected CaR-HEK cells were used as a model to further examine the signalling cross-talk between CaR and cAMP-generating compounds.
Figure 4.3. Effect of increasing \( \text{Ca}^{2+} \) concentrations on ERK phosphorylation in CaR-HEK cells co-transfected with EP4R.

Panel A) Representative immunoblots showing the effect of increasing \( \text{Ca}^{2+} \) concentrations (0.5-5 mM) on ERK phosphorylation in CaR-HEK cells (i) and in CaR-HEK cells co-transfected with EP4R (ii). Panel B) Quantification of the responses are shown as sigmoidal dose response curves. Data was normalised to the maximal response. (ns) not-significant vs. control EC50 by paired t test (two-tailed); n=6 from 3 independent experiments.
4.3.4 Effect of the $G_{\alpha}$ agonist PGE$_2$ on CaR-induced Ca$^{2+}$i mobilisation

4.3.4.1 Effect of PGE$_2$ on CaR-induced Ca$^{2+}$i mobilisation as a function of concentration

It has been verified that co-expression of EP$_4$R in CaR-HEK cells did not enhance CaR-induced Ca$^{2+}$i mobilisation or affect ERK phosphorylation in the absence of exogenous agonist (Figure 4.1 and 4.3). Thus, it was necessary to next investigate whether increasing intracellular cAMP concentrations through the activation of endogenous EP$_4$R using the prostanoid agonist PGE$_2$ alters CaR activity as determined by increasing Ca$^{2+}$i mobilisation. Therefore, Fura-2 loaded CaR-HEK cells were incubated in 2 mM Ca$^{2+}$o-containing buffer then exposed to increasing concentrations of PGE$_2$ (0.1-100 nM). This was done to confirm whether PGE$_2$ can enhance CaR response in a concentration-dependent manner and if so, to identify the EC$_{50}$ for PGE$_2$. Data quantification showed that 10 nM PGE$_2$ significantly increased CaR-induced Ca$^{2+}$i mobilisation versus 0.1 and 1 nM (Figure 4.4A). Increasing the PGE$_2$ concentration further to 100 nM, was without additional effect (ns), thus confirming that 10 nM is the optimal concentration for use. The Hill coefficient for the sigmoidal dose response curve fitted as described in (section 2.14) is 0.5 (Figure 4.4B). Also, the EC$_{50}$ for PGE$_2$ (0.4 ± 0.1 nM) was calculated from the sigmoidal concentration-effect curve (Figure 4.4B).
Figure 4.4. Effect of PGE₂ on CaR-induced Ca²⁺ mobilisation in CaR-HEK as a function of concentration.

Panel A) Representative trace showing Ca²⁺ changes (Fura-2 ratio) in 2 single cells (orange, blue) or “global” cluster of cells (black) exposed to 2 mM Ca²⁺₀ followed by increasing PGE₂ concentration (0.1-100 nM). Panel B) Concentration-effect curve showing % maximal response, defined as area under the curve/min, against the PGE₂ concentration. (ns) not-significant, *p<0.05 and **p<0.001 vs. 10 nM PGE₂ by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=5 from 3 independent experiments.
4.3.4.2 Effect of PGE$_2$ on CaR-induced Ca$^{2+}$i mobilisation as a function of extracellular calcium concentration

Next, to determine whether PGE$_2$ increases CaR sensitivity to Ca$^{2+}$o concentration, calcium imaging was performed on CaR-HEK cells exposed to increasing Ca$^{2+}$o concentrations in the presence or absence of 10 nM PGE$_2$. From the representative traces, in PGE$_2$-treated cells the incremental increase in Ca$^{2+}$o concentration buffers from 0.5 through 2, 3, 5 and 10 mM caused cumulative CaR-induced Ca$^{2+}$i mobilisation and the responses to 2, 3 and 5 mM Ca$^{2+}$o were substantially enhanced relative to control (Figure 4.5A). However, PGE$_2$ did not significantly alter the maximal response at 10 mM Ca$^{2+}$o ($p=0.4$ by Mann-Whitney test). From the sigmoidal concentration effect curves in Figure 4.5B, the EC$_{50}$ value (2.5 ± 0.1) for Ca$^{2+}$o in PGE$_2$-treated CaR-HEK cells was significantly lower in comparison to the EC$_{50}$ (4.0 ± 0.3) for the control group. The Hill coefficient for the sigmoidal dose response curves fitted as described in (section 2.14) are 3.5 for control and 5.4 for PGE$_2$ (Figure 4.5B). This suggests that cotreatment with PGE$_2$ significantly enhances CaR-induced signalling in CaR-HEK cells.
Figure 4.5. PGE₂ enhances Ca²⁺₀-induced Ca²⁺₁ mobilisation in CaR-HEK cells.

Panel A) Representative traces showing Ca²⁺₁ changes (Fura-2 ratio) in 2 single cells (orange, blue) or “global” cluster of cells (black) in response to increasing Ca²⁺₀ concentrations (0.5-10 mM) in control CaR-HEK cells (i) or plus PGE₂ (10 nM) (ii). Panel B) Quantification of the responses are shown as sigmoidal dose response curves. Data was normalised to the maximal response. ***P<0.001 vs. control EC₅₀ by unpaired t test (two-tailed); n=8 from 3 independent experiments.
4.3.4.3 Effect of PGE$_2$ on CaR- induced Ca$^{2+}$\textsubscript{i} mobilisation at subthreshold Ca$^{2+}$\textsubscript{o} concentration

The minimum Ca$^{2+}$\textsubscript{o} concentration necessary to initiate Ca$^{2+}$\textsubscript{i} mobilisation is referred to as the threshold concentration and thus any concentration just below that is a subthreshold concentration. Previously in our lab, it was observed that this subthreshold Ca$^{2+}$\textsubscript{o} concentration is in the range 1.6 - 1.8 mM Ca$^{2+}$\textsubscript{o} for Ca$^{2+}$\textsubscript{i} mobilisation in CaR-HEK cells (Campion, 2013). Therefore, next it was examined whether PGE$_2$ could lower the Ca$^{2+}$\textsubscript{o} concentration needed to initiate Ca$^{2+}$\textsubscript{i} mobilisation.

In this experiment, Fura-2 loaded CaR-HEK cells were exposed to 1.8 mM Ca$^{2+}$\textsubscript{o}-containing buffer then PGE$_2$ (10 nM) was further added to the buffer resulting in a significant increase in Ca$^{2+}$\textsubscript{i} mobilisation (Figure 4.6A). However, to confirm that this PGE$_2$ effect is due to the activation of CaR and not to a direct effect of PGE$_2$, the calcilytic NPS-2143 (Cx; 1 µM) was subsequently added to the buffer. Quantification of the AUC of the global cell responses confirmed that PGE$_2$ did significantly increase the CaR-induced Ca$^{2+}$\textsubscript{i} at a subthreshold Ca$^{2+}$\textsubscript{o} concentration, and, that this effect was significantly inhibited by subsequent cotreatment with a calcilytic (*P<0.05 vs. 1.8 mM Ca$^{2+}$\textsubscript{o} + PGE$_2$ by repeated measures one-way ANOVA with Dunnett’s multiple comparisons test; Figure 4.6B). In confirmation of this was the observation in other experiments, not shown, that PGE$_2$ was without effect on Ca$^{2+}$\textsubscript{i} mobilisation when the Ca$^{2+}$\textsubscript{o} concentration was only 0.5 mM.
Figure 4.6. Effect of PGE₂ and NPS-2143 on subthreshold Ca²⁺₀ induced Ca²⁺ᵢ mobilisation in CaR-HEK cells.

Panel A) Representative trace showing Ca²⁺ᵢ changes (Fura-2 ratio) in 2 single cells (orange, blue) or "global" cluster of cells (black) exposed to PGE₂ (10 nM) followed by NPS-2143 (1 µM) in subthreshold Ca²⁺₀. Panel B) Quantification of changes in Ca²⁺ᵢ mobilisation as area under the curve/min. Data was normalised to the maximal response and normality was confirmed by Shapiro-Wilks test. Data presented as mean ± sem. *P<0.05 vs. 1.8 mM Ca²⁺₀ + PGE₂ by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=5 from 3 independent experiments.
4.3.5 Effect of the $G\alpha_s$ agonist isoprenaline on CaR-induced $Ca^{2+}_i$ mobilisation

4.3.5.1 Effect of isoprenaline on CaR-induced $Ca^{2+}_i$ mobilisation as a function of concentration

Having shown that PGE$_2$ increases the sensitivity of CaR-induced Ca$^{2+}_i$ mobilisation, it was then necessary to determine whether activation of other endogenously-expressed $G\alpha_s$-coupled GPCRs may elicit the same effect. Thus, in this experiment, the $\beta$-adrenoceptor agonist isoprenaline was used to activate AC and thus increase intracellular cAMP generation. This question is of specific interest here since isoprenaline has been shown to increase PTH secretion in bovine parathyroid cells, an effect attenuated by cotreatment with the $\beta$-blocker propranolol (Brown et al., 1977b). Moreover, the gene microarrays mentioned previously (Table 3.2) identified the $\beta_2$ adrenergic receptor as being amongst the 16,000 genes likely to be expressed in bovine and murine PTG. To determine the concentration dependence of isoprenaline on CaR-induced Ca$^{2+}_i$ mobilisation, Fura-2 loaded CaR-HEK cells were incubated in 2 mM Ca$^{2+}_o$-containing buffer and then exposed to increasing isoprenaline concentration (0.1, 1, 10 and 100 nM). Data quantification showed that 10 nM isoprenaline significantly increased CaR-induced Ca$^{2+}_i$ mobilisation versus 0.1 and 1 nM (Figure 4.7A). Increasing the isoprenaline concentration further to 100 nM, was without additional effect (ns), this confirming that 10 nM is the optimal concentration to use. For reference, the $EC_{50}$ for isoprenaline was $4.4 \pm 1.3$ nM as calculated from the resulting sigmoidal concentration-effect curve (Figure 4.7B).
Figure 4.7. Effect of isoprenaline on CaR-induced Ca\textsuperscript{2+} mobilisation in CaR-HEK as a function of concentration.

Panel A) Representative trace showing Ca\textsuperscript{2+}\textsubscript{i} changes (Fura-2 ratio) in 2 single cells (orange, blue) or” global” cluster of cells (black) exposed to 2 mM Ca\textsuperscript{2+}\textsubscript{o} followed by increasing isoprenaline concentration (0.1-100 nM). Panel B) Concentration-effect curve showing % maximal response, defined as area under the curve/min, against the isoprenaline concentration. (ns) not-significant, **p<0.01 and ***p<0.001 vs. 10 nM isoprenaline by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=10 from 3 independent experiments.
4.3.5.2 Effect of isoprenaline on CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation as a function of extracellular calcium concentration

Next, to determine whether isoprenaline increases the sensitivity of CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation, intracellular calcium imaging was performed on CaR-HEK cells exposed to increasing Ca\textsuperscript{2+}\textsubscript{o} concentrations in the presence or absence of 10 nM isoprenaline. From the representative traces, in isoprenaline-treated cells, the incremental increase in Ca\textsuperscript{2+}\textsubscript{o} concentrations from 0.5 through 2, 3, 5 and 10 mM caused cumulative CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation and these responses were substantially enhanced relative to control (Figure 4.8A). From the sigmoidal concentration-effect curves in Figure 4.8B, the EC\textsubscript{50} value (2.7 ± 0.2 mM) for Ca\textsuperscript{2+}\textsubscript{o} in isoprenaline co-treated CaR-HEK cells was significantly lower in comparison to the control EC\textsubscript{50} (4.0 ± 0.3) in the absence of isoprenaline. This leftward shift in the concentration-effect curve for Ca\textsuperscript{2+}\textsubscript{o} in isoprenaline co-treated cells indicates that β-adrenoceptor activation enhanced CaR sensitivity. Indeed, the maximal response at 10 mM Ca\textsuperscript{2+}\textsubscript{o} was also significantly increased in isoprenaline-treated cells (*p=0.045). The Hill coefficient for the sigmoidal dose response curves fitted as described in (section 2.14) are 3.5 for control and 4.9 for isoprenaline (Figure 4.8B).
Figure 4.8. Isoprenaline enhances Ca\textsuperscript{2+}\textsubscript{o}–induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation in CaR-HEK cells.

Panel A) Representative traces showing Ca\textsuperscript{2+}\textsubscript{i} changes (Fura-2 ratio) in 2 single cells (orange, blue) or ”global” cluster of cells (black) in response to increasing Ca\textsuperscript{2+}\textsubscript{o} concentrations (0.5-10 mM) in CaR-HEK cells (i) or plus isoprenaline (10 nM) (ii). Panel B) Quantification of the responses are shown as sigmoidal dose response curves. Data was normalised to the maximal response. **P<0.01 vs. control EC\textsubscript{50} by unpaired t test (two-tailed); n=8 from 3 independent experiments.
4.3.5.3 Effect of isoprenaline on CaR-induced $Ca^{2+}\textsubscript{i}$ mobilisation at subthreshold $Ca^{2+}\textsubscript{o}$ concentration

Again, in order to confirm that isoprenaline lowers the $Ca^{2+}\textsubscript{o}$ concentration needed to initiate CaR-induced $Ca^{2+}\textsubscript{i}$ mobilisation in CaR-HEK cells, cells were exposed to isoprenaline (10 nM) at a subthreshold $Ca^{2+}\textsubscript{o}$ concentration (1.8 mM) as previously described in result 4.3.4.3. Indeed, the isoprenaline induced $Ca^{2+}\textsubscript{i}$ mobilisation in a similar fashion to the PGE$_2$ tested previously. Then, as before, the calcilytic NPS-2143 (1 µM) was further added to the buffer to confirm that the isoprenaline effect was due to the activation of CaR as opposed to a direct effect of the isoprenaline. The reproducibility of these observations was confirmed by quantification of the AUC of the global cell responses which showed that isoprenaline significantly increased the CaR-induced $Ca^{2+}\textsubscript{i}$ mobilisation at subthreshold $Ca^{2+}\textsubscript{o}$ concentrations (**p<0.01) and that this effect was significantly inhibited by the subsequent addition of the calcilytic (*p<0.05) (Figure 4.9B). In other experiments, not shown, isoprenaline was without effect on $Ca^{2+}\textsubscript{i}$ mobilisation when the $Ca^{2+}\textsubscript{o}$ concentration was only 0.5 mM.
Figure 4.9. Effect of isoprenaline and NPS-2143 on subthreshold Ca\textsuperscript{2+}\textsubscript{o} induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation in CaR-HEK cells.

Panel A) Representative trace showing Ca\textsuperscript{2+}\textsubscript{i} changes (Fura-2 ratio) in 2 single cells (orange, blue) or “global” cluster of cells (black) exposed to isoprenaline (10 nM) followed by NPS-2143 (1 µM) in subthreshold Ca\textsuperscript{2+}\textsubscript{o}. Panel B) Quantification of changes in Ca\textsuperscript{2+}\textsubscript{i} mobilisation as area under the curve/min. Data was normalised to the maximal response and presented as mean ± sem. \*P<0.05 and \**p<0.01 vs. 1.8 mM Ca\textsuperscript{2+}\textsubscript{o} + isoprenaline by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=6 from 3 independent experiments.
4.3.6 Effect of the $\text{G}_{\alpha_s}$ agonist histamine on CaR-induced $\text{Ca}^{2+}_i$ mobilisation

4.3.6.1 Effect of histamine on CaR-induced $\text{Ca}^{2+}_i$ mobilisation as a function of concentration

As explained in the introduction, high levels of histamine can be found in adenomatous or hyperplastic PTG tissues (Abboud et al., 1981). Moreover, the histamine 1 receptor (H$_1$R) and its ligand synthase (histidine decarboxylase) genes were identified as probably-expressed genes in the microarray analysis (Table 3.2) of cDNA obtained from bovine PTG tissue. Thus, it was necessary to examine the effect of histamine as a potential cAMP generator on CaR activity as assessed by measuring $\text{Ca}^{2+}_i$ mobilisation. In this experiment, Fura-2 loaded CaR-HEK cells were incubated in 2 mM $\text{Ca}^{2+}_o$-containing buffer and then exposed to increasing histamine concentrations 30, 100, 300 and 1000 nM. Data quantification showed that 300 nM significantly increased the CaR-induced $\text{Ca}^{2+}_i$ mobilisation relative to the effect of 30 nM histamine. However, 100 nM and 1 µM responses were not statistically different from 300 nM response (Figure 4.10B) by Friedman test with post Dunn’s test. The histamine EC$_{50}$ (30.3 ± 1.3 nM) was calculated from the histamine sigmoidal concentration-effect curve (Figure 4.10B).
Figure 4.10. Effect of histamine on CaR-induced Ca\textsuperscript{2+} \textsubscript{i} mobilisation in CaR-HEK as a function of concentration.

Panel A) Representative trace showing Ca\textsuperscript{2+} \textsubscript{i} changes (Fura-2 ratio) in 2 single cells (orange, blue) or "global" cluster of cells (black) exposed to 2 mM Ca\textsuperscript{2+} \textsubscript{o} followed by increasing histamine concentration (30-1000 nM). Panel B) Concentration-effect curve showing % maximal response, defined as area under the curve/min, against the histamine concentration. (ns) not-significant and **P<0.01 vs. 300 nM histamine by Friedman test with Dunn’s post-test; n=6 from 3 independent experiments.
4.3.6.2 Effect of histamine on CaR-induced Ca\textsuperscript{2+} mobilisation as a function of extracellular calcium concentration

Next, to determine whether histamine increases the sensitivity of CaR-induced Ca\textsuperscript{2+} mobilisation, intracellular calcium imaging was performed on CaR-HEK cells exposed to increasing Ca\textsuperscript{2+}o concentrations in the presence or absence of 1 µM histamine. From the representative traces, in histamine-treated cells, the incremental increase in [Ca\textsuperscript{2+}]\textsubscript{o} from 0.5 through 2, 3 and 5 mM caused cumulative CaR-induced Ca\textsuperscript{2+}i mobilisation (Figure 4.11A). However, from the sigmoidal concentration-effect curves, the EC\textsubscript{50} value (5.2 ± 0.6 mM) for Ca\textsuperscript{2+}o in histamine co-treated CaR-HEK cells was not statistically different from the EC\textsubscript{50} (5.3 ± 0.4) for the untreated cells (Figure 4.11B). This suggested that despite the observation in Figure 4.10, histamine co-treatment did not appear to alter the sensitivity of CaR-induced Ca\textsuperscript{2+}i mobilisation. It should be noted however, that one possible explanation for this discrepancy is that the histamine did elicit acute histamine receptor activation but that this became rapidly desensitised before the concentration-effect curve reached its threshold concentration. The Hill coefficient for the sigmoidal dose response curves fitted as described in (section 2.14) are 2.3 for control and 2.8 for histamine (Figure 4.11B).
Figure 4.11. Effect of histamine on Ca\textsuperscript{2+}_o–induced Ca\textsuperscript{2+}_i mobilisation in CaR-HEK cells.

Panel A) Representative traces showing Ca\textsuperscript{2+}_i changes (Fura-2 ratio) in 2 single cells (orange, blue) or "global" cluster of cells (black) in response to increasing Ca\textsuperscript{2+}_o concentrations (0.5-10 mM) in CaR-HEK cells (i) or plus histamine (1 µM) (ii). Panel B) Quantification of the responses are shown as sigmoidal dose response curves. Data was normalised to the maximal response. (ns) not-significant vs. control EC\textsubscript{50} by unpaired t test (two-tailed); n=7-11 from 3 independent experiments.
4.3.6.3 Effect of histamine on CaR-induced Ca\textsuperscript{2+}i mobilisation at subthreshold Ca\textsuperscript{2+}o concentration

In order to test whether histamine does lower the Ca\textsuperscript{2+}o concentration needed to initiate CaR-induced Ca\textsuperscript{2+}i mobilisation in CaR-HEK cells (despite the apparent lack of difference in EC\textsubscript{50} shown in Figure 4.11), cells were exposed to histamine (1 µM) at a subthreshold Ca\textsuperscript{2+}o concentration (1.8 mM) as previously described in section 4.3.4.3. The calcilytic NPS-2143 (1 µM) was then added to the buffer to confirm that the histamine effect, if present, was occurring due to the activation of CaR. Indeed, quantification of the AUC of the global cell responses confirmed that histamine had significantly increased the CaR-induced Ca\textsuperscript{2+}i mobilisation at subthreshold Ca\textsuperscript{2+}o concentration (***p<0.001) and further that this effect was significantly inhibited by the subsequent addition of the calcilytic (***p<0.001) (Figure 4.12B). This confirms that upon addition, histamine elicits an immediate increase in CaR sensitivity, although whether this effect is sustained for the duration of a 20 minutes concentration-effect experiment is less clear.
Figure 4.12. Effect of histamine and NPS-2143 on subthreshold Ca\(^{2+}\)\(_o\) induced Ca\(^{2+}\)\(_i\) mobilisation in CaR-HEK cells.

Panel A) Representative trace showing Ca\(^{2+}\)\(_i\) changes (Fura-2 ratio) in 2 single cells (orange, blue) or ”global” cluster of cells (black) exposed to histamine (1 µM) followed by NPS-2143 (1 µM) in subthreshold Ca\(^{2+}\)\(_o\). Panel B) Quantification of changes in Ca\(^{2+}\)\(_i\) mobilisation as area under the curve/min. Data was normalised to the maximal response and presented as mean ± sem. ***/P<0.001 vs. 1.8 mM Ca\(^{2+}\)\(_o\) + histamine by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=6 from 3 independent experiments.
4.3.7 PGE$_2$ treatment enhances protein kinase A activity using anti-PKA phospho-substrate antibody in CaR-HEK cells exposed to moderate Ca$^{2+}$o concentrations

Having established that agonists of 3 different G$\alpha$-coupled receptors are capable of elevating CaR sensitivity, I next tested what would be the effect of PGE$_2$ treatment on protein kinase A activity in CaR-HEK cells exposed to moderate Ca$^{2+}$ concentrations. Therefore, I used a phospho-PKA substrate (RRXS/T) monoclonal antibody to examine whether PKA-mediated phosphorylation can be enhanced by PGE$_2$ in moderate Ca$^{2+}$o concentrations. It should be noted that the identity of the bands shown here are unknown, the antibody simply detects proteins phosphorylated on that PKA consensus sequence and thus serves as an indirect assay.

In this experiment, the CaR-HEK cells were incubated for 20 minutes in 0.5 mM Ca$^{2+}$-containing buffer for equilibration then, cells were incubated in 0.5, 3 or 5 mM Ca$^{2+}$ in the presence or absence of PGE$_2$ for 10 minutes. After that, cells were lysed with RIPA buffer and immunoblotted against anti-PKA phospho-substrate antibody as described in methodology section 2.3. From the representative immunoblot in figure 4.13A, PGE$_2$ treatment in 0.5, 3 and 5 mM Ca$^{2+}$ increased phosphorylation of a number of proteins between 30 and 100 kDa. Data quantification by densitometry showed that PGE$_2$ co-treatment significantly enhanced PKA substrate phosphorylation in 0.5 and 3 mM Ca$^{2+}$ (*p<0.05) but was without additional effect in 5 mM Ca$^{2+}$ (Figure 4.13B). This result suggested that subsequent treatment with PGE$_2$ enhances more phosphorylation of PKA substrate which indicates that PGE$_2$ treatment did cause acute PKA activation and thus indirectly suggested that it had elicited cAMP generation.
Figure 4.13. Effect of PGE$_2$ on PKA substrate phosphorylation in CaR-HEK cells exposed to moderate Ca$_{2+}$ concentrations.

Panel A) Representative immunoblot for CaR-HEK cells exposed to moderate Ca$_{2+}$ concentrations in presence or absence of PGE$_2$ (10 nM) then processed for immunoblotting using anti-PKA phospho-substrate antibody. Panel B) Quantification of changes in PKA substrate phosphorylation levels as % maximal response. (ns) not-significant and *p<0.05 by paired t test (two-tailed); n=4 from 3 independent experiments.
4.3.8 Effect of the PKA inhibitor H89 on PGE$_2$-induced PKA activation in CaR-HEK cells treated with low Ca$^{2+}_o$ concentration

Having established that PGE$_2$ enhances PKA substrate phosphorylation, the selective PKA inhibitor H89 was used to investigate whether the effect of PGE$_2$ definitely occurred via PKA or not, since other kinases might be able to phosphorylate those sites. Therefore, CaR-HEK cells were incubated in buffer containing 0.5 mM Ca$^{2+}_o$ and PGE$_2$ in the presence or absence of H89. From the representative immunoblot, cotreatment with H89 inhibited the PKA substrate phosphorylation induced by PGE$_2$ (Figure 4.14A). Quantification of these data by densitometry confirmed that PGE$_2$ had significantly increased the PKA substrate phosphorylation at 0.5 mM Ca$^{2+}_o$ (****$p<0.0001$) and that this effect was significantly inhibited by the subsequent addition of H89 (****$p<0.0001$; repeated measures ANOVA with Dunnett’s multiple comparisons post-test; Figure 4.14B). This suggested that the positive functional effect of PGE$_2$ on PKA substrate phosphorylation is indeed PKA-dependent.
Figure 4.14. Effect of the PKA inhibitor H89 on PGE2-induced PKA activation in CaR-HEK cells.

Panel A) Representative immunoblot for CaR-HEK cells treated with PGE2 (10 nM) in the presence or absence of H89 (10 μM) in 0.5 mM Ca$^{2+}_o$ then processed for immunoblotting using anti-PKA phospho-substrate antibody. Panel B) Quantification of changes in PKA substrate phosphorylation levels as % maximal response. ****p<0.0001 by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=12 from 4 independent experiments.
4.3.9 PGE$_2$ treatment potentiates extracellular signal-regulated kinase (ERK) phosphorylation and inhibits P70 S6 kinase in CaR-HEK cells exposed to moderate Ca$^{2+}_o$ concentrations

CaR activation is well known to increase ERK1/2 phosphorylation and p38$^{\text{MAPK}}$ in CaR-HEK cells (Kifor et al., 2001; Conigrave and Ward, 2013). Furthermore, previously in our lab it has been found that activation of CaR also enhanced the phosphorylation of other kinases including IGFR (Tyr1131), IκBα (Ser32) and P70 S6 kinase (Thr389) (Bin-Khayat, 2016). Therefore, to further investigate the crosstalk between CaR activation and cAMP-generating compounds, in this experiment I attempted to examine the effect of PGE$_2$ on ERK1/2 and P70 S6 kinase phosphorylation in CaR-HEK cells treated with moderate Ca$^{2+}_o$ concentrations. These two kinases were selected based on a preliminary experiment which tested the effect of PGE$_2$ on different kinases including ERK1/2, P38$^{\text{MAPK}}$, IGFR, IκBα and P70 S6 kinase in CaR-HEK cells exposed to moderate Ca$^{2+}_o$ concentrations (not shown).

Thus in this experiment, CaR-HEK cells were exposed to increasing Ca$^{2+}_o$ concentrations (0.5, 2, 3 and 5 mM) in the absence or presence of PGE$_2$ (10 nM) and then the phosphorylation level of ERK1/2 and P70 S6 kinase was determined by semi-quantitative immunoblotting. As shown in Figure 4.15A, raising Ca$^{2+}_o$ concentrations from 0.5 to 5 mM markedly increased the phosphorylation level of ERK1/2 and P70 S6 kinase. However, cotreatment with PGE$_2$, significantly increased ERK1/2 activation but inhibited P70 S6 kinase phosphorylation (Figure 4.15A). Quantification of these changes by densitometry demonstrated that PGE$_2$ significantly increased ERK1/2 phosphorylation in 0.5, 2 and 3 mM Ca$^{2+}_o$ (***P<0.001) but had no further effect in 5 mM Ca$^{2+}_o$ (Figure 4.15B). In contrast, cotreatment with PGE$_2$ significantly inhibited P70 S6 kinase phosphorylation in 0.5, 2 and 5 mM Ca$^{2+}_o$ (****P<0.0001, *P<0.05 and **p<0.01) although it had no effect in 2 mM Ca$^{2+}_o$(Figure 4.15B). Together, these data suggested that there is significant crosstalk between CaR and Ga$_{a}$-GPCRs in CaR-HEK cells and further raises the possibility of signal bias i.e. where PGE$_2$ exerts different effects on different signalling pathways. The Hill coefficient for the sigmoidal dose response curves fitted as described in (section 2.14) are 5.6 for control and 7.3 for PGE$_2$ (Figure 4.15Bi) versus 5.9 for control and 8.0 for PGE$_2$ in (Figure 4.15Bii).
Figure 4.15. PGE$_2$ treatment potentiates extracellular signal-regulated kinase (ERK) phosphorylation and inhibits P70 S6 kinase in CaR-HEK cells exposed to moderate Ca$_{o}^{2+}$ concentrations.

Panel A) Representative immunoblots showing the effect of (10 nM) PGE$_2$ on ERK (Ai) and P70 S6 kinase (Aii) phosphorylation in CaR-HEK cells lysate exposed to increasing Ca$_{o}^{2+}$ concentrations (0.5-5 mM). Panel B) Quantification of PGE$_2$ responses on ERK (Bi) and P70 S6 kinase (Bii) phosphorylation levels as % maximal response. (ns) not-significant, *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 by Wilcoxon test (paired) two-tailed; n= 19 from 6 independent experiments.
4.3.10 Selective EP<sub>4</sub>R antagonist inhibits PGE<sub>2</sub>-induced ERK phosphorylation in CaR-HEK cells

Having confirmed that PGE<sub>2</sub> enhanced ERK1/2 phosphorylation in CaR-HEK, it was then necessary to investigate via which receptor the PGE<sub>2</sub> mediated this response. Therefore, CaR-HEK cells were incubated for 10 minutes at 37°C in 0.5 mM Ca<sup>2+</sup><sub>o</sub> and PGE<sub>2</sub> (10 nM)-containing buffer in the absence or presence of either the selective EP<sub>4</sub>R antagonist; L-161982 (1 μM) or the broad-spectrum EP-R antagonist; AH6809 (30 μM). Then, cells were lysed and processed for immunoblotting against p-ERK antibody. From the representative immunoblot shown in Figure 4.16A, PGE<sub>2</sub> increased the phosphorylation level of ERK1/2 and this response was inhibited significantly by cotreatment with the selective EP<sub>4</sub>R antagonist L-161982. Quantification of the relative ERK phosphorylation by densitometry, confirmed that PGE<sub>2</sub> significantly increased ERK phosphorylation in 0.5 mM Ca<sup>2+</sup><sub>o</sub> (**p<0.01) and that this effect was significantly inhibited using the selective EP<sub>4</sub>R antagonist L-161982 (**p<0.01) but was largely unaffected by AH6809 at least when using 30 μM (Figure 4.16B). This result indicates that PGE<sub>2</sub> mediated ERK activation in CaR-HEK cells via activation of EP<sub>4</sub>R, the receptor detected in PT cells (Chapter 3).
Figure 4.16. Selective EP\textsubscript{4}R antagonist inhibits PGE\textsubscript{2}-induced ERK phosphorylation in CaR-HEK cells.

Panel A) Representative immunoblot for CaR-HEK cells incubated in 0.5 mM Ca\textsuperscript{2+} with PGE\textsubscript{2} (10 nM) in the presence of L-161982 (1 µM) or AH6809 (30 µM) then processed for immunoblotting using anti-pERK antibody. β-actin was used as a loading control. Panel B) Data quantification of changes in ERK phosphorylation levels as % maximal response. (ns) not-significant, **P<0.01, ***P<0.001 by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=8 from 4 independent experiments.
4.3.11 The selective $\beta_1$ adrenergic receptor antagonist atenolol fails to significantly inhibit isoprenaline-induced ERK phosphorylation in CaR-HEK cells

Having shown that PGE$_2$ can increase ERK phosphorylation at low Ca$_{\text{out}}^{2+}$ concentrations and identified the receptor by which it occurs, it was then necessary to investigate if the G$\alpha$-agonist isoprenaline elicits the same effect. In addition, the selective $\beta_1$ adrenergic receptor antagonist atenolol was used to examine whether the isoprenaline-mediated ERK phosphorylation was occurring via activation of the $\beta_1$ adrenergic receptor. Thus in this experiment, CaR-HEK cells were incubated for 10 minutes at 37°C in 0.5 mM Ca$_{\text{out}}^{2+}$ and isoprenaline (10 nM)-containing buffer in the presence of either 1 $\mu$M or 10 $\mu$M atenolol. Then, cells were lysed and processed for immunoblotting against the pERK antibody. From the representative immunoblot shown in Figure 4.17A, isoprenaline clearly increased the phosphorylation level of ERK in 0.5 mM Ca$_{\text{out}}^{2+}$. Quantification of ERK phosphorylation by densitometry confirmed that isoprenaline significantly increased ERK phosphorylation in 0.5 mM Ca$_{\text{out}}^{2+}$ (*p<0.05). With regards whether the effect was mediated via the $\beta_1$ adrenergic receptor or not, there was no statistically significant decrease in ERK phosphorylation following atenolol cotreatment meaning that it cannot be concluded that the $\beta_1$ adrenergic receptor was responsible (Figure 4.17B). It should be noted however that there was a concentration-dependent trend which may be indicative of $\beta_1$AR involvement but with a large variance within the data it is impossible to conclude anything either way. Nevertheless, this result suggests that isoprenaline induces ERK activation in CaR-HEK cells even in low Ca$_{\text{out}}^{2+}$ concentrations.
Figure 4.17. Effect of the selective β1-adrenergic receptor antagonist atenolol on isoprenaline-induced ERK phosphorylation in CaR-HEK cells.

Panel A) Representative immunoblot for CaR-HEK cells incubated in 0.5 mM Ca\(^{2+}\)\(_o\) with isoprenaline (10 nM) in the presence of either 1 μM or 10 μM atenolol then processed for immunoblotting using anti-pERK antibody. β-actin was used as a loading control. Panel B) Quantification of changes in ERK phosphorylation levels as % maximal response. (ns) not-significant and *P<0.05 by Friedman test with Dunn’s multiple comparisons post-test; n=5 from 3 independent experiments.
4.3.12 The selective H₁R antagonist pyrilamine inhibits histamine-induced ERK phosphorylation in CaR-HEK cells

Having demonstrated that both PGE₂ and isoprenaline increase ERK activation in low Ca²⁺₀ concentration in CaR-HEK cells, it was necessary to investigate whether histamine produces the same effect. The H₁R and H₂R selective antagonists were both used to examine through which histamine receptor subtype and histamine-mediated ERK phosphorylation may be occurring. In this experiment, CaR-HEK cells were incubated for 10 minutes at 37°C in 0.5 mM Ca²⁺₀ and histamine (1 µM) in the presence of either the H₁R-selective antagonist pyrilamine (1 µM) or the H₂R-selective antagonist ranitidine (1 µM). Then, cells were lysed and processed for immunoblotting against the pERK antibody. From the representative immunoblot shown in Figure 4.18A, histamine appeared to increase the phosphorylation level of ERK and this response was inhibited with the selective H₁R antagonist pyrilamine. Quantification of the relative ERK phosphorylation confirmed that histamine significantly increased ERK phosphorylation in 0.5 mM Ca²⁺₀ (**p<0.01) and that this effect was significantly inhibited with the selective H₁R antagonist pyrilamine (*p<0.05) but was completely unaffected by the selective H₂R antagonist ranitidine (Figure 4.18B). This result indicates that histamine mediated ERK activation in CaR-HEK cells via H₁R.
Figure 4.18. Selective H₁R antagonist inhibits histamine-induced ERK phosphorylation in CaR-HEK cells.

Panel A) Representative immunoblot for CaR-HEK cells incubated in 0.5 mM Ca²⁺ₒ with histamine (1 µM) in the presence of either pyrilamine (1 µM) or ranitidine (1 µM) then processed for immunoblotting using anti-pERK antibody. β-actin was used as a loading control. Panel B) Quantification of changes in ERK phosphorylation levels as % maximal response. (ns) not-significant, *P<0.05 and **P<0.01 by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=6 from 3 independent experiments.
4.3.13 The additive effect of adding very low concentrations of several Ga\(_s\) agonists on CaR-induced Ca\(_{2+}\) mobilisation in CaR-HEK cells

Finally, it has been shown in this chapter that the activation of any of at least 3 different Ga\(_s\)-coupled GPCRs endogenously expressed in CaR-HEK cells elicits increased CaR sensitivity. However, when performing CaR experiments \textit{in vitro} we would normally seek to remove all other confounding signals and examine just the CaR response using pharmacological concentrations of agonist or drug. However, \textit{in vivo} it is likely to be the case that cells are continuously exposed to multiple agonists and thus physiological CaR responses may actually involve the integration of multiple inputs to produce a specific response. To demonstrate the proof of principle of this idea, CaR-HEK cells were cotreated simultaneously with several Ga\(_s\) GPCR agonists but using subthreshold concentrations of the treatments when tested on their own. In the control experiment, Fura-2 loaded cells were incubated in 2 mM Ca\(_{2+}\)o-containing buffer and then exposed in turn to very low concentrations of PGE\(_2\) (1 pM), followed by isoprenaline (10 pM) and finally histamine (1 nM). Cells were returned to agonist-free 2 mM Ca\(_{2+}\)o buffer prior to the subsequent treatment. What was observed was that on their own none of the agonists elicited any increase in CaR sensitivity at the low concentrations tested.

However, in the following experiment cells were again incubated in 2 mM Ca\(_{2+}\)o-containing buffer but then exposed to a combined cocktail of all 3 agonists, namely 1 pM PGE\(_2\), 10 pM isoprenaline and 1 nM histamine. From the representative traces shown in Figure 4.19A, it would appear that the Ga\(_s\) GPCR agonist cocktail elicited a robust increase in CaR-induced Ca\(_{2+}\) mobilisation. Quantification of the responses as area under the curve confirmed that the addition of the cocktail significantly enhanced CaR-induced Ca\(_{2+}\) mobilisation (**p<0.01) by paired t-test. In contrast, the responses of agonists added on their own were not statistically different from the 2 mM Ca\(_{2+}\)o response (p>0.05; repeated measures one-way ANOVA with post Dunnett’s multiple comparisons test; Figure 4.19B). This result suggests that a combination of very low concentrations of Ga\(_s\)-agonists may be sufficient to produce an additive effect capable of eliciting significantly enhanced CaR-induced Ca\(_{2+}\) mobilisation.
Figure 4.19. The additive effect of several Gαs agonists on CaR-induced Ca\(^{2+}\) mobilisation in CaR-HEK cells.

Panel A) Representative traces showing Ca\(^{2+}\) changes (Fura-2 ratio) in 2 single cells (orange, blue) or “global” cluster of cells (black) in response to 2 mM Ca\(^{2+}\)\(_o\) plus individual small doses of Gαs-agonists (1 pM PGE\(_2\), 10 pM isoprenaline or 1 nM histamine) in CaR-HEK cells (i) or plus cocktail containing all 3 agonists (ii). Panel B) Quantification of changes in Ca\(^{2+}\) mobilisation as area under the curve/min. Data was normalised to the maximal response and presented as mean ± sem. (ns) not-significant and **P<0.01 by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test or paired t test; n=6-8 from 3-4 independent experiments.
4.4 Discussion

4.4.1 The hypothesis of intrinsic parathyroid hormone secretion

Our central hypothesis is that the spontaneous secretion of PTH depends on locally-produced factors such as prostanoids or histamine that activate $\text{G}_\alpha$-coupled receptors in an autocrine/paracrine fashion to increase cAMP production and thus elicit the PTH release (Conigrave, 2016). The endocrine “long-loop” negative feedback mechanism involved in the inhibition of PTH secretion via $\text{Ca}^{2+}$-induced CaR activation is reasonably well understood. However, our hypothesis includes the possibility that there may be another autocrine “short-loop” inhibitory mechanism that could acutely limit PTH release in the event of a larger transient increase in intracellular cAMP levels. That is, any transient increase in cAMP levels could potentiate $\text{Ca}^{2+}$-induced CaR activation which in turn would suppress PTH secretion by lowering cAMP levels through the activation of $\text{G}_{\alpha_0}$ (Fitzpatrick et al., 1986) or a $\text{Ca}^{2+}$-calmodulin dependent PDE (Conigrave and Ward, 2013) (Figure 4.20A). This proposed short-loop inhibition would dampen excess PTH secretion occurring during the period while the PTH takes effect. The presence of such short-loop feedback might provide a good explanation for the normal ultradian pulsatile pattern of PTH secretion (Harms et al., 1989) reviewed in (Chiavistelli et al., 2015) (Figure 4.20B). While not able to prove or disprove this hypothesis directly, the data presented in this chapter do at least represent proof of the principle that a range of $\text{G}_\alpha$-coupled GPCRs are capable of cross-talking with CaR signalling and may even combine to do so.

Having confirmed the expression of $\text{EP}_4$R and the enzymes required for the synthesis of PGE$_2$ in bovine PT gland tissue (Sections 3.3.2 and 3.3.4) and then demonstrated that PGE$_2$ enhances CaR-induced $\text{Ca}^{2+}_i$ mobilisation (Section 3.3.5) at least in CaR-HEK cells, there may be some merit to our hypothesis. Moreover, functional data from our collaborator Professor Arthur Conigrave (Sydney) demonstrated that inhibition of COX2 (the rate-limiting enzyme in prostaglandin production), or, treatment with either the broad-spectrum prostanoid receptor inhibitor AH6809 or the selective $\text{EP}_4$R antagonist L-161,982 acutely and reversibly reduced PTH
secretion from perifused human PT cells exposed to low Ca$_{2+}^\circ$ (1.0 mM) (Szczawinska et al., 2015).

4.4.2 Using CaR-HEK cells as a model to study the crosstalk between CaR and other GPCRs

In the absence of a suitable human parathyroid cell line, HEK cells stably transfected with CaR represent the best alternative model for studying CaR pharmacology and signalling (Ward, 2004). Although we can obtain bovine parathyroid glands, the recovery of healthy PT cells is far too low to permit us to perform the molecular experiments we wished to undertake. Other limitations include the fact that, i) bovine CaR lacks the PKA phosphorylation sites present in human CaR and thus may signal differently where cAMP is involved, and ii) there is significant inconsistency between bovine PT cells preparations including both bacterial contamination and with fibroblasts or other non-chief cells, iii) overnight downregulation of the CaR and iv) resistance to transfection with siRNA or plasmids (Campion, 2013). Nevertheless, it will be essential to compare, at the earliest opportunity, these signalling data obtained from cells in which the CaR is overexpressed with data obtained from cells in which the CaR is expressed endogenously (Ward, 2004).
Figure 4.20. Schematic diagram proposing how cAMP may elicit self-limiting PTH secretion.

Panel A) Activation of Gαs-linked (EP4, IP and H2) receptors increase cAMP and CaR-induced Ca2+ mobilisation. ↑Ca2+ suppresses cAMP and PTH release from the cell through activation of Ca2+-calmodulin dependent PDE (short loop). PTH increase Ca2+ absorption from bone and kidney raising blood Ca2+o concentration and subsequently CaR activation (long loop). Panel B) short loop feedback explains normal pulsatile PTH secretion. In the case of ↑Ca2+o, a sharp decrease in PTH secretion occur (long loop feedback).
4.4.3 Effect of EP₄R cotransfection on CaR function in CaR-HEK cells

It was reported in 1985 that PTG cells may produce prostaglandins including PGE₂, PGF₂α and thromboxane β₂ (Brown and Swartz, 1985). Furthermore, there was a report that PGE₂ stimulates both cAMP production and PTH release in dispersed bovine PT cells (Gardner et al., 1978; Gardner et al., 1980). It is well known that EP₂ and EP₄ receptors couple to Ga₅ upon PGE₂ binding thus stimulating AC with a resultant increase in intracellular cAMP levels (Carboneau et al., 2017), though EP₄R may also couples to phosphatidylinositol 3-kinase (PI3K) through Gaₒ (Fujino and Regan, 2006; Sugimoto and Narumiya, 2007).

In 1992, (Kjelsberg et al.) first drew attention to the agonist-independent constitutive activity of GPCRs when they found that mutations in the ICD of the α₁β-adrenergic receptor resulted in an increase of the constitutive activity of the receptor (Milligan, 2003; Costa and Cotecchia, 2005). Then regarding the EP₄R, Fujino et al. (2002) demonstrated that this particular receptor also exhibit constitutive activity and can elicit greater T-cell factor (Tcf)/β-catenin signalling (an important pathway involved in colon cancer development) as compared with wild-type or EP₂R-transfected HEK cells. However, it has been mentioned that EP₄R stimulates Tcf/β-catenin transcriptional activity mainly through Gaₒ-coupling (Fujino et al., 2002). Also, Hawcroft et al. (2007) noted that EP₄R exhibits constitutive activity in HT-29 human colon cancer cells when it is overexpressed in those cells by stable transfection. Having demonstrated that EP₄R coexpression in CaR-HEK cells did not enhance CaR- induced Ca²⁺ mobilisation (Figure 4.1) and that indomethacin did not decrease such Ca²⁺ mobilisation in response to elevated Ca²⁺ (Figure 4.2), this suggests that EP₄R is not constitutively active and thus that it still needs the addition of exogenous agonist. Moreover, it was shown that EP₄R coexpression in CaR-HEK cells did not enhance the CaR-induced ERK phosphorylation supporting the idea that the activation of EP₄R in CaR-HEK cells is ligand-dependent (Figure 4.3). It should be noted however that given the transient transfection method used, there may be cell-to-cell and day-to-day variability in transfection efficiency. Therefore some cells in the supposedly “transfected” category may not have expressed substantive EP₄R expression and perhaps cotransfection with a fluorescent marker for example would have allowed the selection of high expression cells only.
4.4.4 Parathyroid hormone secretion between $G\alpha_s$ and $G\alpha_{q/11}$ mediated signalling

Before the discovery of CaR, it was clear that increased intracellular cAMP levels are linked to PTH hormone secretion in vivo. For example, it has been shown that cAMP-generating compounds including isoproterenol, dopamine and PGE$_2$ stimulate the release of PTH in bovine PT cells (Brown et al., 1977a; Brown et al., 1977b; Gardner et al., 1978). Moreover, in 1984, it was found that forskolin also increases cAMP level and elicits PTH release in dispersed bovine PTG cells (Shoback and Brown, 1984).

In PT gland, CaR inhibits PTH secretion via activation of $G\alpha_{q/11}$ which leads to increased Ca$^{2+}$i mobilisation (Shoback and Brown, 1984; Shoback et al., 1988). In CaR-HEK cells, raised Ca$^{2+}$o concentrations leads to CaR activation which in turn again stimulates $G\alpha_{q/11}$/PLC signalling. PLC activation leads to generation of IP$_3$ and DAG from PIP$_2$. Then, IP$_3$ binds to the IP$_3$ receptor present on the surface of ER resulting in the release of Ca$^{2+}$i to the cytoplasm (Conigrave and Ward, 2013; Hannan et al., 2017). Both DAG and Ca$^{2+}$i activate PKC which in turn phosphorylate the receptor at different sites, while phosphorylation of CaR$_{T888}$ leads to receptor inactivation and thus inhibits CaR-induced Ca$^{2+}$i mobilisation. Then, the receptor quickly dephosphorylates again by the action of a calyculin-sensitive protein phosphatase (most likely PP2A) allowing the reactivation of the receptor and mobilisation of Ca$^{2+}$i again (Davies et al., 2007; McCormick et al., 2010). Of note it has been known for some time that the activation of PP2A might be cAMP and/or PKA dependent (Davare et al., 2000; Ahn et al., 2007). This cycle of phosphorylation and dephosphorylation provides a reasonable potential explanation for oscillatory Ca$^{2+}$i mobilisation. CaR activation with high Ca$^{2+}$o concentration stimulates the protein phosphatase PP2A which in turn maintains the receptor in the dephosphorylated state and thus enabling sustained Ca$^{2+}$i mobilisation (McCormick, 2008; Conigrave and Ward, 2013).

Gerbino et al. in 2005 reported that cAMP significantly enhanced the frequency and the amplitude of CaR-induced Ca$^{2+}$i oscillations in CaR-HEK cells loaded with Fura-2. It has been reported that increased cAMP levels could influence Ca$^{2+}$i via PKA-dependent phosphorylation of either IP$_3$ receptor present in the surface of ER or via the plasma membrane Ca$^{2+}$-ATPase (PMCA) responsible for Ca$^{2+}$ clearance (Bruce
et al., 2003). Here, it has been demonstrated that cAMP-generating compounds including PGE₂ and isoprenaline enhance CaR-induced Ca²⁺ᵢ mobilisation in a concentration-dependent manner, and, lowers the EC₅₀ for Ca²⁺₀ to induce Ca²⁺ᵢ mobilisation thus supporting and extending the Gerbino et al. findings (Sections 4.3.4 and 4.3.5).

4.4.5 Effect of cAMP in CaR-mediated Ca²⁺ᵢ mobilisation in subthreshold Ca²⁺₀ concentration

CaR couples to a wide range of G proteins including Gaα₁₁, Gaα₁₂, Ga₁₂/₁₃ and even in some cancer cell types to Gaα (Conigrave and Ward, 2013; Conigrave, 2016). Ca²⁺ᵢ mobilisation is used as a readout of Gaα₁₁ signalling whereas, plasma membrane (PM) ruffling is used as a measure of Ga₁₂/₁₃ and β-arrestin pathways (Davey et al., 2011). Interestingly, Ca²⁺₀ induces PM ruffling at lower concentrations, with an EC₅₀ between 1.5-2 mM which is lower than for Ca²⁺₀-induced Ca²⁺ᵢ mobilisation which has an EC₅₀ that is around 3.3-4.2 mM Ca²⁺₀ (Davies et al., 2006). Thus, the “subthreshold” Ca²⁺₀ concentrations used here, which were around 1.8 mM Ca²⁺₀, are capable of enhanced actin polymerisation but not Ca²⁺ᵢ mobilisation or ERK phosphorylation. It has been suggested that the absence of Ca²⁺ᵢ mobilisation at low Ca²⁺₀ concentrations is due to PKC-dependent CaR phosphorylation. That is, inhibition of PKC using GF109203X promotes Ca²⁺ᵢ mobilisation at these subthreshold Ca²⁺₀ concentrations (Conigrave and Ward, 2013). However as mentioned earlier, PGE₂ or isoprenaline-induced cAMP generation also enhances Ca²⁺ᵢ mobilisation at lower Ca²⁺₀ concentrations (Gerbino et al., 2005; Figures 4.6 and 4.9). In addition, these findings are consistent with findings of previous study in this laboratory (Campion, 2013) which reported that increasing cAMP with forskolin, PTx or IBMX lowers the threshold for CaR to induce Ca²⁺ᵢ mobilisation and that forskolin certainly lowers the EC₅₀ for Ca²⁺₀ overall. The key point however is that for every Gaα GPCR agonist that could increase Ca²⁺ᵢ mobilisation in CaR-HEK cells, this effect could be significantly inhibited using the calcilytic NPS-2143 proving that the effect occurs via an action on CaR and not directly. This is interesting because it raises the possibility that there may be other instances where activation of a GPCR appears to enhance Ca²⁺ᵢ mobilisation whereas it might in fact be altering cAMP levels instead. This is partly why it is so important to study one
pathway at a time *in vitro* but to always remember that under physiological conditions, multiple pathways may be partially activated and thus interacting with one another.

### 4.4.6 Effect of histamine on CaR activation

Histamine can activate four histamine receptors subtypes (H₁-H₄) via stimulation of different G protein-mediated intracellular signalling pathways. H₁R is known to activate Ga₉/₁₁/PLC signalling pathway causing increased Ca²⁺ mobilisation (Seifert *et al.*, 2013; van Unen *et al.*, 2016a). H₂R is known to activate Ga₆ protein resulting in increased intracellular cAMP levels (Panula *et al.*, 2015). Whereas, H₃R and H₄R couple to PTx-sensitive Gaᵢ/o (Bahre and Kaever, 2017). In the bovine gene microarray (Table 3.2), H₁R and its ligand synthase were detected among the 16,000 likely-to-be-expressed genes. Iwata *et al.* (2005) reported that HEK-293 cells appear to express H₁R endogenously as histamine induces Ca²⁺ mobilisation in fura-2 loaded cells and that the effect was blocked with the H₁R blocker pyrilamine. In contrast, van Unen *et al.* (2016a) mentioned that in CaR-HEK cells transfected with Epac (cAMP reporter), histamine addition increased cAMP levels and that this response was antagonised with the selective H₂R antagonist (ranitidine) suggesting that CaR-HEK cells also express H₂R endogenously.

Having demonstrated that histamine enhanced the sensitivity of CaR-induced Ca²⁺ mobilisation (Figure 4.10) and that histamine lowered the threshold for CaR to induce Ca²⁺ mobilisation (Figure 4.12) in CaR-HEK cells, it is clear that at the very least, the cells express histamine receptor endogenously.

A similar stimulatory effect of histamine on CaR-induced signalling was seen (Figure 4.18) for ERK phosphorylation in 0.5 mM Ca²⁺₀ and interestingly this response was significantly inhibited using the selective H₁R blocker (pyrilamine) suggesting that the ERK response at least is mediated via H₁R. This supports the finding of a previous study which found that histamine induces ERK phosphorylation via H₁R exclusively in HeLa cells (Jain *et al.*, 2016) and that it did so via the PLCβ, PKCδ and MEK pathway (Jain *et al.*, 2016).

Interestingly, while histamine did enhance CaR-induce Ca²⁺ᵢ mobilisation in 2 mM Ca²⁺₀-containing buffer, it did not lower the EC₅₀ in the Ca²⁺₀ concentration-effect
curve (Figure 4.11). Since these two results are largely incompatible, this raises the possibility that in the case of the latter experiment the histamine receptors are desensitised rapidly upon agonist binding (Hishinuma and Ogura, 2000; Raspe et al., 2016). This idea is consistent with the data of van Unen et al. (2016a) who suggested that activation of H₁R causes a transient increase in Ca²⁺, suggesting that the receptor undergoes fast desensitisation.

4.4.7 Effect of PGE₂ as a cAMP generator in PKA activation in CaR-HEK cells exposed to moderate Ca²⁺o concentrations

Cyclic AMP is a well-known second messenger which has two main downstream effectors; PKA and Epac. It has been mentioned that CaR has two PKA phosphorylation sites (S-899 and S-900) located in its arginine rich domain in the proximal C-terminus domain (Garrett et al., 1995; Stepanchick et al., 2010). However, Stepanchick et al. (2010) demonstrated that S-899 is important for increasing the abundance of CaR in the cell membrane but had no effect on CaR functioning. Moreover, Riccardi et al. (1995) reported that rat kidney CaR has two PKA phosphorylation sites whereas, bovine parathyroid CaR has no PKA phosphorylation sites suggesting that cAMP might modulate function of CaR in rat kidney but not in bovine PTG and thus would not be a fundamental regulatory mechanism even if true in rodents. On the other hand, Bösel et al. (2003) demonstrated that PKA inhibitors slightly inhibited CaR-mediated IP₃ signalling in CaR-HEK cells but it has a synergistic effect when it added to the PKC inhibitor suggesting that these two signalling pathways might influence each other.

Here I demonstrated that PGE₂ significantly enhanced PKA substrate phosphorylation in CaR-HEK cells incubated in 0.5 or 3 mM Ca²⁺o (Figure 4.13), and that this effect was completely abolished with the PKA inhibitor (H89) (Figure 4.14) suggesting that PGE₂ does indeed positively modulate PKA activation. This finding is consistent with Campion (2013) who showed that forskolin also significantly enhanced PKA phosphorylation in CaR-HEK cells in 1.8 and 3 mM Ca²⁺o. However, Campion (2013) demonstrated that forskolin-enhanced CaR-induce Ca²⁺i mobilisation effect was not significantly inhibited by PKA inhibitors (H89 or KT-5720) and neither was it affected by mutation of either of the CaR’s PKA sites S-899 and S-900. These questions raise the issue of how exactly cAMP does
enhance CaR-mediated \( \text{Ca}^{2+} \) mobilisation and in this regard Taylor (2017) has suggested that cAMP may in some circumstances act directly on the IP\(_3\)R independently of PKA (or Epac) and this might be interesting to test in a future study.

### 4.4.8 Biased signalling arising from activation of \( \text{Ga}_q \) and \( \text{Ga}_{q/11} \)-coupled GPCRs

The two most common readouts of CaR activity employed in laboratories are changes in \( \text{Ca}^{2+} \) mobilisation (or sometimes just IP\(_3\) generation), and, the degree of ERK1/2 phosphorylation. However, it has been demonstrated previously in our lab that CaR activation also mediates the phosphorylation of other kinases including IGF1R, IκBα and P70 S6 kinase. Indeed, it has been shown that P70 S6 kinase is more sensitive to changes in \( \text{Ca}^{2+} \)o concentrations. That is, the EC\(_{50}\) for \( \text{Ca}^{2+} \)o to induce P70 S6 kinase phosphorylation (2.6 ± 0.5) is lower than the EC\(_{50}\) for \( \text{Ca}^{2+} \)o to induce ERK phosphorylation (4.1 ± 0.5) (Bin-Khayat, 2016). Moreover, it has been demonstrated that PKC\( \alpha \) knockdown results in increased CaR-induced \( \text{Ca}^{2+} \)i mobilisation and decreased the level of ERK and P70 S6 kinase phosphorylation. This suggests that \( \text{Ca}^{2+} \)i mobilisation and ERK activation pathways can be dissociated and that PKC\( \alpha \) knockdown targets cellular effectors and not only the phosphorylation of CaR\( ^{T888} \). This appears to represent an example of biased CaR signalling (Bin-Khayat, 2016).

Then with regards the effect of PGE\(_2\) on CaR signalling, PGE\(_2\) cotreatment did potentiate ERK1/2 phosphorylation but in contrast tended to inhibit P70 S6 kinase in CaR-HEK cells exposed to moderate \( \text{Ca}^{2+} \)o concentrations (Result 4.3.9). On one level these data suggest simply that there is significant crosstalk between CaR and the \( \text{Ga}_q \)-coupled receptor (EP\(_4\)R). The identity of the PGE\(_2\) receptor was specifically shown in 4.3.10 to be EP\(_4\)R given the inhibitory effect of the selective EP\(_4\)R antagonist L-161982. This finding is consistent with what has been reported by Li et al. (2017) that the EP\(_4\)R-selective antagonist completely inhibited the PGE\(_2\)-mediated ERK activation in Tca8113 cells. However, perhaps the more interesting finding was that whereas PGE\(_2\) could enhance ERK phosphorylation (and \( \text{Ca}^{2+} \)i mobilisation for that matter), it had no positive effect on P70 S6 kinase activity and in fact was inhibitory at most concentrations tested. This suggests that crosstalk from
a second receptor may not necessarily enhance or reduce signalling from the first receptor in a consistent manner but may do so differentially. Such bias means that it cannot always be assumed that the signals induced by a particular receptor in a reductionist experimental design will necessary be the same as those induced in a physiological situation where other GPCRs are signalling concomitantly.

Regarding, the observation that EP₄R enhanced ERK phosphorylation it should be noted that this has been reported in different cell types. For example, Yamaguchi et al. (2000) has reported that PGE₂ induced ERK activation in polycystic kidney epithelium, while Pozzi et al. (2004) reported that PGE₂ or the selective EP₄R agonist PGE₁-OH induced ERK activation via PI3K in mouse colon adenocarcinoma (CT26) cells. Fujino et al. (2003) reported that PGE₂ induced PI3K/ERK activation via EP₄R in HEK-293 cells stably transfected with the receptor. Also, Nandi et al. (2017) has reported that PGE₂ or an EP₄R agonist increased PI3K-dependent ERK phosphorylation in rat mesenteric lymphatic endothelial cells (RMLECs). Chang et al. (2015) also mentioned that EP₄R play an important role in colon tumour growth via ERK phosphorylation. Thus, the finding in the current study appears consistent with this literature.

Then regarding the suppressed effect of S6 kinase, Scott et al. (1996) reported that in bovine airway smooth muscle cells, AC activation with forskolin inhibited P70 S6 kinase activity in time-dependent manner. In contrast, Dufour et al. (2014) has reported that PGE₂ enhances mTORC1/6S kinase activation via EP₄R in LS174T cells. Also, Okunishi et al. (2014) reported that PGE₂ mediated ribosomal protein S6 kinase activation and mTOR inhibition in normal adult lung fibroblast and mouse peritoneal macrophages (PMs). Thus it is not clear why CaR-induced P70 S6 kinase activity was inhibited rather than enhanced by PGE₂ cotreatment in the current experiments.

Finally, isoprenaline significantly enhanced ERK phosphorylation in 0.5 mM Ca²⁺₀, however, the selective β₁-adrenoceptor blocker (atenolol) failed to inhibit isoprenaline-induced ERK phosphorylation suggesting that this response is not mediated via the β₁ adrenoceptor. Daaka et al. (1998) reported that in HEK-293 cells β₂-adrenergic receptor mediates ERK activation. Moreover, it has been reported that in microarray analysis performed to detect mRNA levels of different GPCRs in
HEK-293 cells, the β2 adrenoceptor but not the β1 receptor was likely expressed in HEK-293 cells (Atwood et al., 2011).

4.4.9 The combined stimulatory effect arising from limited stimulation of multiple GPCRs

Finally, it was demonstrated that combined treatment with agonists for EP4R, adrenoceptor and histamine receptor exerted a stimulatory effect on CaR-induced Ca\(^{2+}\) mobilisation. This result suggests that there is an additive effect of all Ga\(_s\) agonists and that they exhibit a similar effect on enhancing CaR-mediated Ca\(^{2+}\) mobilisation. Indeed, one might speculate that such cotreatment may reflect more closely the \textit{in vivo} reality of receptor signalling, where there are more influencing factors which may influence cAMP levels and subsequently affect the release of PTH.

In support of my result there are numerous studies reported such additive effect of multiple signals in normal physiology for example, in 3T3-L1 adipocytes, the PGF\(_{2\alpha}\) act with cAMP in a synergistic way to enhance glucose transport via increased GLUT1 expression and PKC activation (Chiou and Fong, 2004). Also, Serpa et al. (2015) reported that in hippocampus, adenosine (A1) and cannabinoid (CB1) receptors combined their inhibitory effects on cAMP levels in an additive manner. Moreover, Zhang et al. (2016) reported that the tryptophan derivative, L-1,2,3,4-tetrahydronorharman-3-carboxylic acid (TNCA) act as a co-agonist with Mg\(^{2+}\) in enhancing CaR-induced Ca\(^{2+}\) oscillations and ERK1/2 activation.

4.5 Summary

The data presented in this chapter suggest that there is significant crosstalk between CaR and Ga\(_s\) GPCRs. That is, increasing cytosolic cAMP accumulation enhances CaR-mediated Ca\(^{2+}\) mobilisation in response to moderate Ca\(^{2+}\) concentrations that on their own are insufficient to elicit Ga\(_{q/11}\)-mediated Ca\(^{2+}\) oscillations. I have also demonstrated here that increasing cAMP levels particularly with PGE\(_2\) or isoprenaline reduces the EC\(_{50}\) for Ca\(^{2+}\) to induce Ca\(^{2+}\) mobilisation. Also, having demonstrated that CaR-induced P70 S6 kinase activity was inhibited rather than
enhanced by PGE$_2$ cotreatment suggests the existence of differential crosstalk mechanisms between CaR and Ga$_s$ GPCRs that may allow for biased signalling.
CHAPTER 5

CaR-Mediated Modulation of Cyclic AMP Levels in HEK-293 Cells and Human Bronchial Smooth Muscle Cells
5.1 Introduction

As detailed earlier, PTH secretion in PT cells has been associated with increased cAMP generation via the activation of endogenously expressed Ga\(_s\)-coupled GPCRs (Brown et al., 1977a; Brown et al., 1977b; Gardner et al., 1978; Shoback and Brown, 1984). Then CaR activation is understood to oppose this effect however it is less clear precisely how CaR reduces cAMP levels, that is whether through a Ga\(_s\) dependent increase in Ca\(_{2+}\)\(_i\) concentration activating a Ca\(_{2+}\)\(_i\)-dependent phosphodiesterase, or, through the classical Ga\(_i\)-mediated intracellular signalling pathway (Bruce et al., 2003; Gerbino et al., 2005). This chapter will investigate the crosstalk between CaR and the Ga\(_s\)-mediated signalling pathway using a fluorescence resonance energy transfer (FRET)-based cAMP sensor based on Epac (Klarenbeek et al., 2015). This technique permits investigation of the inhibitory effect of CaR on cAMP production in real time in single living CaR-HEK cells. The particular significance of this is that having shown that Ga\(_s\)-mediated cAMP production enhances CaR-induced Ca\(_{2+}\)\(_i\) mobilisation, now the reverse effect of CaR activity on cAMP levels can be determined. While this is interesting from a purely signalling perspective, what is ultimately intended is to see how CaR signalling interacts with cAMP signalling in physiological and indeed in pathological pathways.

It has been reported by Yarova et al. (2015) that CaR is expressed in both human and mouse airway smooth muscle (ASM) cells and that the expression of CaR is increased in asthmatic patients or in sensitised animals. Also, it has been reported that increased production of cationic proteins including eosinophilic cationic protein (ECP) and compounds such as spermine, spermidine and putrescine as well as the elevation of Ca\(_{2+}\)\(_o\) concentration in the inflamed tissue are directly linked to the pathogenesis and the severity of asthma (Kurosawa et al., 1992; Meurs et al., 2003; North et al., 2013). Yarova et al (2015) reported that in ASMs, polycation-mediated CaR activation leads to increased Ca\(_{2+}\) mobilisation, inhibited cAMP production and increased p38\(^{MAPK}\) phosphorylation and it has been shown that the CaR-inhibiting calcilytics oppose these effects. Therefore, the calcilytics might be considered as a new drug for asthma with potentially fewer systemic side effects compared to bronchodilators and steroids. The treatment regimen for an acute asthma attack
usually includes a β2-adrenoceptor agonist (such as salbutamol), and thus it would be interesting to test the combined effect of CaR action and inhibition (with a calcilytic) on salbutamol signalling. Thus, in the latter part of the chapter, BSMCs were used to investigate the crosstalk between the β2-agonist (salbutamol) and a calcilytic as determined by Ca\(^{2+}\) mobilisation and the phosphorylation levels of different kinases to see whether these two drugs mediate the same signalling pathways or antagonise each other in some cases.

5.2 Materials and Methods

HEK-293 cells were cultured and transiently transfected with cAMP reporter as described in sections 2.6 and 2.7 respectively. FRET was performed as described in section 2.13. BSMCs were cultured as described in section 2.6. Cell assay and immunoblotting were performed as described in sections 2.8 and 2.3 respectively.

5.3 Results

5.3.1 H187 vector transfection into HEK-293 cells and validation of PGE\(_2\)-mediated cAMP accumulation in CaR-HEK cells using FRET

In order to establish the FRET technique in the current laboratory and to assess the production of cAMP upon PGE\(_2\) stimulation, live cell measurements were performed in CaR-HEK cells transiently transfected with the H187 cAMP (Epac) sensor (Klarenbeek et al., 2015). During the experiment, the coverslips were placed in a perfusion chamber and the cells were exposed to experimental buffer containing 0.5 mM Ca\(^{2+}\) followed by buffer supplemented with PGE\(_2\) (10 nM) to increase cAMP levels. The total experiment time was 8 minutes and images were acquired every 5 seconds using an Olympus IX83 inverted microscope as detailed in methodology section (2.13). 48-hours after transfection, the fluorescent protein was detected in the cytoplasm of the transfected cells, with some nuclear signal apparent though whether this was cytoplasmic signal in front of or behind the nucleus was not further tested (Figure 5.1A). The addition of the PGE\(_2\) elicited an increase in CFP emission and concomitant decrease in YFP emission as shown in (Figure 5.1B). Changes in FRET ratio (i.e. the changes of CFP to YFP) reflect the alteration in cAMP levels such that
association of cAMP results in loss of FRET. That is, the higher the cytosolic cAMP concentration the greater the FRET loss. Strictly speaking, the FRET change should be reported as a decrease, however, I felt it was conceptually simpler to report the CFP:YFP ratio instead such that an increase in the ratio indicates the equivalent increase in cAMP.

Figure 5.1. Altered CFP/YFP emission ratio in CaR-HEK cells expressing the H187 cAMP sensor in response to PGE$_2$.

Panel A) Wild type HEK-293 cells expressing Epac sensor imaged 48-hours after transfection using Olympus IX83 inverted microscope. Panel B) Representative trace showing that PGE$_2$ (10 nM) stably increases intracellular cAMP levels, expressed as a higher FRET ratio (i.e. altered emission intensity in the cyan CFP and yellow YFP channels; black trace = CFP/YFP ratio) in CaR-HEKs cells cotransfected with the cAMP reporter.
5.3.2 Effect of increasing Ca\(^{2+}\) concentrations on PGE\(_2\)-induced cAMP production in CaR-HEK cells transfected with cAMP reporter

It has been reported that CaR activation suppresses cAMP production via \(\alpha_{i/o}\) mediated signalling or via the inhibitory effect of Ca\(^{2+}\) on cAMP (Conigrave and Ward, 2013). By taking advantage of this sensitive cAMP reporter (H187), I was able to examine the effect of CaR activation on cAMP signalling in CaR-HEK cells. Therefore, CaR-HEK cells transiently transfected with the cAMP FRET sensor were stimulated with PGE\(_2\) (10 nM) and the response then antagonised by the addition of buffers containing increasing Ca\(^{2+}\) concentrations (3 and 5 mM). Each treatment lasted only for approximately two minutes to allow its specific response to develop. The CFP and YFP emission signals were recorded and the FRET ratio was calculated as CFP/YFP emission in selected area of a single cell using ImageJ software as described in section 2.1.3. From the representative trace showing CFP/YFP ratio in Figure 5.2A, the PGE\(_2\)-induced elevation of cAMP (expressed as a higher FRET ratio) was slightly inhibited with 3 mM Ca\(^{2+}\) in some cells whereas, the 5 mM Ca\(^{2+}\) inhibitory effect was more profound and persistent in all cells. Quantification of the changes in FRET ratio amplitudes between different conditions confirmed that PGE\(_2\) significantly increased cAMP production and that 5 mM Ca\(^{2+}\) significantly reversed the PGE\(_2\)-induced cAMP formation effect (*p<0.05 vs. 0.5 mM Ca\(^{2+}\) + PGE\(_2\)) by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test. This result, confirmed that high Ca\(^{2+}\) concentration is a potent inhibitor of cAMP accumulation in CaR-HEK cells (Figure 5.2B).
Figure 5.2. Increasing Ca\textsuperscript{2+} concentrations reverse PGE\textsubscript{2}-induced cAMP production in CaR-HEK cells.

Panel A) Representative trace showing that addition of high Ca\textsuperscript{2+} concentrations (3 and 5 mM) attenuate PGE\textsubscript{2}-induced cAMP formation in CaR-HEK cells cotransfected with the Epac vector. Panel B) Quantification of changes in FRET ratio as % maximal response. (ns) not-significant and *p<0.05 vs. 0.5 mM Ca\textsuperscript{2+} + PGE\textsubscript{2} by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=10 from 4 independent experiments.
5.3.3 The CaR positive allosteric modulator R568 enhanced CaR-mediated cAMP inhibition in CaR-HEK cells transfected with cAMP reporter

In order to confirm that it was specifically CaR activation that was responsible for high Ca\(^{2+}\)\(_i\)-induced attenuation of PGE\(_2\)-mediated cAMP production, it was necessary to use the CaR-specific positive allosteric modulator R568, in place of 5 mM Ca\(^{2+}\)\(_i\) in both CaR-HEK and wild-type HEK-293 cells. Thus, in this experiment either CaR-HEK or HEK-293 cells not expressing CaR were transiently transfected with Epac vector with cells then exposed to PGE\(_2\) (10 nM) until reaching the peak of PGE\(_2\)-induced cAMP formation. Then, cells were exposed to buffer containing 3 mM Ca\(^{2+}\)\(_i\) for 2 minutes followed by subsequent addition of R568 (1 µM). From the representative traces showing CFP/YFP ratio in Figure 5.3A, a fast drop in FRET ratio was observed after addition of the R568 in CaR-HEK cells but was without effect in wild-type HEK-293 cells lacking the CaR. Quantification of the changes in FRET ratio amplitudes between different conditions confirmed that the PGE\(_2\) significantly raised the cAMP levels expressed as an increase in FRET ratio both in CaR-HEK and wild type HEK-293 cells (**p<0.01 or ***p<0.001). However, subsequent addition of the calcimimetic (R568) in 3 mM Ca\(^{2+}\)\(_i\) significantly reversed the PGE\(_2\)-induced cAMP increase in CaR-HEK cells but was without effect on wild-type cells. ***p<0.001 and (ns) not-significant vs. 0.5 mM Ca\(^{2+}\)\(_i\) + PGE\(_2\) by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test (Figure 5.3B). This result, confirmed that the CaR allosteric modulator R568 enhances the CaR-mediated inhibitory effect on cAMP in CaR-HEK cells but not in wild-type HEK-293 cells.
Figure 5.3. The CaR positive allosteric modulator R568 enhanced CaR-mediated cAMP inhibition in CaR-HEK cells.

Panel A) Representative traces showing that subsequent addition of R568 to 3 mM Ca\textsuperscript{2+}o-containing buffer attenuated PGE\textsubscript{2}-induced cAMP formation in CaR-HEKs cells cotransfected with the Epac vector but was without effect in wild-type HEK-293 cells. Panel B) Quantification of changes in FRET ratio as % maximal response. (ns) not-significant. **p<0.01 and ***p<0.001 vs. 0.5 mM Ca\textsuperscript{2+}o + PGE\textsubscript{2} by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=12 from 4 independent experiments.
5.3.4 Effect of the PKC inhibitor GF102903X on CaR-induced cAMP inhibition

5.3.4.1 Effect of PKC inhibition on CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation
Before examining the effect of the PKC inhibitor GF102903X on CaR-induced cAMP inhibition using the FRET based cAMP biosensor in CaR-HEK cells, I first confirmed that in my hands GF102903X enhances CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation as previously reported (Davies et al., 2007; McCormick et al., 2010). This effect is believed to occur by preventing the PKC-mediated inhibition of CaR signalling via CaR\textsuperscript{T888} phosphorylation. Therefore, in this experiment Fura-2 loaded CaR-HEK cells were exposed to 3 mM Ca\textsuperscript{2+}\textsubscript{o}-containing buffer to first induce oscillatory Ca\textsuperscript{2+}\textsubscript{i} mobilisation. Then, 300 nM GF102903X was further added to the buffer. From the representative trace shown in Figure 5.4A, as expected GF102903X enhanced the response of the cells to Ca\textsuperscript{2+}\textsubscript{o} determined as high-frequency oscillations and even sustained Ca\textsuperscript{2+}\textsubscript{i} mobilisation. Quantification of the AUC of the global cell responses confirmed that the GF102903X significantly increased the CaR-induced Ca\textsuperscript{2+}\textsubscript{i} concentrations (**p<0.01) by paired t-test (Figure 5.4B).

5.3.4.2 Effect of PKC on CaR-induced cAMP suppression in CaR-HEK cells transfected with cAMP reporter
It is well-known that CaR may activate multiple signalling pathways including G\textsubscript{q/11}, G\textsubscript{s}, G\textsubscript{i/o} and G\textsubscript{12/13} (Conigrave and Ward, 2013). Thus, having established that CaR activation acutely inhibits cAMP production in CaR-HEK cells it is now necessary to determine through which signalling pathway this occurs. As such, the first issue to examine is whether the inhibitory effect of CaR\textsuperscript{T888} phosphorylation applies to cAMP production as well as to Ca\textsuperscript{2+}\textsubscript{i} mobilisation and thus GF102903X was used to inhibit the PKC-mediated CaR phosphorylation (Davies et al., 2007). In this experiment, CaR-HEK cells cotransfected with the cAMP reporter were preincubated with GF102903X (300 nM) in low Ca\textsuperscript{2+}\textsubscript{o}-containing buffer (0.5 mM Ca\textsuperscript{2+}\textsubscript{o}) followed by PGE\textsubscript{2} (10 nM) addition until the cells reached the peak of PGE\textsubscript{2}-induced cAMP production. Then, cells were exposed to PGE\textsubscript{2} along with the PKC inhibitor but now in 3 mM Ca\textsuperscript{2+}\textsubscript{o}-containing buffer to see whether the PKC inhibitor presence enhances CaR-mediated cAMP suppression response and reverses the PGE\textsubscript{2} effect. GF102903X was dissolved in DMSO, so in the control group DMSO vehicle was added instead of GF102903X. From the representative traces showing...
FRET ratio in Figure 5.5A, it appears that GF102903X failed to enhance the CaR inhibitory effect on cAMP production. Quantification of the changes in FRET ratio showed that PGE2 addition significantly increased cAMP levels in both group of cells (***(p<0.0001). Also, 3 mM Ca^{2+} \text{O} plus GF102903X is not statistically different from 0.5 mM Ca^{2+} + PGE2 (p>0.05) in both groups of cells, by repeated measures one-way ANOVA with post Dunnett’s multiple comparisons test. This result suggested that CaR-induced cAMP inhibition is not PKC dependent.
Figure 5.4. PKC inhibition by GF102903X enhances CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation in CaR-HEK.

Panel A) Representative trace showing Ca\textsuperscript{2+}\textsubscript{i} changes (Fura-2 ratio) in 2 single cells (orange, blue) or "global" cluster of cells (black) exposed to 3 mM Ca\textsuperscript{2+}\textsubscript{o} in presence or absence of GF102903X (300 nM) in CaR-HEK cells. Panel B) Quantification of changes in Ca\textsuperscript{2+}\textsubscript{i} mobilisation as area under the curve/min. Data was normalised to the maximal response and presented as mean ± sem. **P<0.01 by paired t-test; n= 5 from 3 independent experiments.
Figure 5.5. PKC inhibition with GF102903X did not enhance the CaR-mediated cAMP suppression in CaR-HEK cells transfected with Epac vector.

Panel A) Representative traces showing that GF102903X addition did not enhance the CaR-mediated cAMP suppression in CaR-HEK cells transfected with the Epac vector relative to the effect in DMSO vehicle-treated cells. Panel B) Quantification of changes in FRET ratio as % maximal response. (ns) not-significant, **p<0.01 and ****p<0.0001 vs. 0.5 mM Ca^{2+} +GF102903X/DMSO +PGE_{2}) by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=10-14 from 4-5 independent experiments.
5.3.5 Effect of the G\(_{\alpha_q/11}\) inhibitor YM-254890 on Ca\(^{2+}\)\(_o\)-induced cAMP suppression in CaR-HEK cells transfected with cAMP reporter

As mentioned before, CaR activation can stimulate multiple signalling pathways and so to further investigate how CaR inhibits cAMP accumulation it was next necessary to examine the effect of the selective G\(_{\alpha_q/11}\) inhibitor YM-254890 to conclude if this inhibitory effect is mediated via the G\(_{\alpha_q/11}\) signalling pathway (perhaps by affecting the activity of a Ca\(^{2+}\)i-modulated PDE). Therefore, in this experiment, CaR-HEK cells cotransfected with Epac (cAMP sensor) were preincubated with YM-254890 (100 nM) in 0.5 mM Ca\(^{2+}\)\(_o\)-containing buffer. Then, PGE\(_2\) was added to increase the cAMP levels followed by the addition of 5 mM Ca\(^{2+}\)\(_o\)-containing buffer in the continued presence of the G\(_{\alpha_q/11}\) inhibitor and PGE\(_2\). YM-254890 was dissolved in DMSO, so in the control group DMSO was added instead of YM-254890. From the representative traces showing FRET ratio in Figure 5.6A, YM-254890 apparently failed to overcome the 5 mM Ca\(^{2+}\)\(_o\)-induced cAMP inhibition relative to the effect of 5 mM Ca\(^{2+}\)\(_o\) in vehicle control cells. Quantification of the changes in FRET ratio amplitudes for each condition demonstrated that the PGE\(_2\) significantly increased cAMP levels in both YM-254890 and DMSO-treated cells (***p<0.001 or ****p<0.0001). On the other hand, the presence of YM-254890 failed to overcome the 5 mM Ca\(^{2+}\)\(_o\)-response (*p<0.05 vs. 0.5 mM Ca\(^{2+}\)\(_o\)+ PGE\(_2\)+ YM-254890/DMSO) by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test. This result suggested that CaR-induced cAMP inhibition is not mediated through the G\(_{\alpha_q/11}\) signalling pathway.
Figure 5.6. Effect of the Gαq/11 inhibitor YM-254890 on high Ca\(^{2+}\)o-induced cAMP suppression in CaR-HEK cells transfected with Epac vector.

Panel A) Representative traces showing that the addition of 5 mM Ca\(^{2+}\)o reversed the effect of PGE\(_2\) on cAMP production in both i) YM-254890 and ii) control (DMSO vehicle-treated) cells. Panel B) Quantification of changes in FRET ratio as % maximal response. (ns) not-significant, *p<0.05, ***p<0.001, ****p<0.0001 vs. 0.5 mM Ca\(^{2+}\)o + Gαq inhibitor/DMSO + PGE\(_2\)) by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=7-11 from 4 independent experiments.
5.3.6 Effect of the Gαi inhibitor PTx on high Ca\textsuperscript{2+\textsubscript{o}}-induced cAMP suppression in CaR-HEK cells transfected with cAMP reporter

Having demonstrated that neither Gα\textsubscript{q/11} inhibition nor PKC inhibition can overcome (or indeed enhance) CaR-mediated cAMP inhibition, next I examined whether CaR suppresses cAMP generation via coupling to Gαi (Brown and MacLeod, 2001; Conigrave and Ward, 2013). For this, CaR-HEK cells cotransfected with Epac sensor were preincubated overnight with pertussis toxin (PTx; 100 ng/ml) which inhibits Gαi through ADP-ribosylation of it (Burns, 1988; van Unen et al., 2016b). Cells were then exposed to increasing Ca\textsuperscript{2+\textsubscript{o}} concentrations (0.5, 3 and 5 mM Ca\textsuperscript{2+\textsubscript{o}}) in the presence of PGE\textsubscript{2} (10 nM). CFP and YFP emission intensities were recorded every 5 seconds during the time course of the experiment and compared to those from the control group (cells not pretreated with PTx). It was found that in PTx-pretreated cells, high Ca\textsuperscript{2+\textsubscript{o}} concentrations failed to attenuate PGE\textsubscript{2}-induced cAMP production (Figure 5.7A) while in the control group high Ca\textsuperscript{2+\textsubscript{o}} concentration (5 mM) reversed the PGE\textsubscript{2} effect (Figure 5.2). Quantification of the changes in FRET ratio amplitudes demonstrated that 5 mM Ca\textsuperscript{2+\textsubscript{o}} significantly lowered the PGE\textsubscript{2} response in the control cells (*p<0.05; Figure 5.2B) whereas there was no significant effect of 5 mM Ca\textsuperscript{2+\textsubscript{o}} in the PTx-pretreated cells (Figure 5.7B). The statistical test used was repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test. Therefore, these data suggest that in CaR-HEK cells, CaR suppresses cAMP accumulation via Gαi/o activation.
Figure 5.7. Effect of the Gαi inhibitor PTx on high Ca\textsuperscript{2+}\textsubscript{o}-induced cAMP suppression in CaR-HEK cells transfected with Epac vector.

Panel A) Representative trace showing that 5 mM Ca\textsuperscript{2+}\textsubscript{o} exerts little effect on PGE\textsubscript{2}-induced cAMP accumulation in PTx-pretreated CaR-HEK cells. Panel B) Quantification of changes in FRET ratio as % maximal response. (ns) not-significant and *p<0.05 vs. 0.5 mM Ca\textsuperscript{2+}\textsubscript{o} + PGE\textsubscript{2} by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=9 from 4 independent experiments.
5.3.7 Effect of salbutamol in Ca$^{2+}$i mobilisation in BSMCs exposed to 2 mM Ca$^{2+}$o concentration

Thus, so far I have demonstrated that in CaR-HEK cells, PGE$_2$-induced cAMP accumulation enhances CaR-induced Ca$^{2+}$i mobilisation while CaR activation antagonises PGE$_2$-induced cAMP accumulation. Therefore, next I examined potential crosstalk between G$\alpha$s signalling and CaR in a cell-type with potential pathological / therapeutic relevance. Salbutamol is a $\beta_2$R agonist used in acute asthma attacks while the calcilytics have been proposed to be novel asthma drugs (Yarova et al., 2015). In order to investigate potential crosstalk between the G$\alpha$s-coupled receptor agonism (salbutamol) and CaR negative allosteric modulation (calcilytic), it was necessary first to examine the effect of salbutamol on CaR activation determined by Ca$^{2+}$i mobilisation. Therefore, in this first experiment, Fura2-loaded BSMCs were exposed to low Ca$^{2+}$o concentration-containing buffer (0.5 mM Ca$^{2+}$o) to establish the baseline 350/380 ratio. Then, cells were stimulated with 2 mM Ca$^{2+}$o resulting in the induction of Ca$^{2+}$i oscillations after which the cells were then cotreated with 1 $\mu$M salbutamol. From the representative trace in Figure 5.8A, it can be seen that 2 mM Ca$^{2+}$o induced Ca$^{2+}$i mobilisation and when salbutamol was further added, the amplitude of the Ca$^{2+}$i oscillations was increased. Quantification of the AUC of the global cell responses confirmed that salbutamol significantly increased the 2 mM Ca$^{2+}$o response (***p<0.001) by paired two-tailed t-test (Figure 5.8B).

Next, in order to confirm that the salbutamol is not enhancing Ca$^{2+}$i mobilisation in low Ca$^{2+}$o buffer, Fura-2 loaded cells were exposed to 0.5 mM Ca$^{2+}$o then, salbutamol (1 $\mu$M) was further added to the buffer. Quantification of the AUC of the global cell responses confirmed that salbutamol did not enhance significantly Ca$^{2+}$i mobilisation in 0.5 mM Ca$^{2+}$o-containing buffer (ns p>0.05) by paired two-tailed t-test (Figure 5.9B) although a small trend change was noted.
Figure 5.8. Salbutamol enhances \( \text{Ca}^{2+}_\text{o} \)-induced \( \text{Ca}^{2+}_\text{i} \) enhancement in human BSM cells.

Panel A) Representative trace showing \( \text{Ca}^{2+}_\text{i} \) changes (Fura-2 ratio) in 2 single cells (orange, blue) or “global” cluster of cells (black) in response to 2 mM \( \text{Ca}^{2+}_\text{o} \) in presence or absence of salbutamol (1 µM) in BSMCs. Panel B) Quantification of changes in \( \text{Ca}^{2+}_\text{i} \) concentrations as area under the curve/min. Data was normalised to the maximal response and presented as mean ± sem. ***P<0.001 by paired t-test (two-tailed); n=12 from 4 independent experiments.
Figure 5.9. Salbutamol failed to significantly enhance $\text{Ca}^{2+}_i$ mobilisation in low $\text{Ca}^{2+}_o$ concentration in BSMCs.

Panel A) Representative trace showing $\text{Ca}^{2+}_i$ changes (Fura-2 ratio) in 2 single cells (orange, blue) or “global” cluster of cells (black) in response to 0.5 mM $\text{Ca}^{2+}_o$ in presence or absence of salbutamol (1 µM). Panel B) Quantification of changes in $\text{Ca}^{2+}_i$ concentrations as area under the curve/min. Data was normalised to the maximal response and presented as mean ± sem. (ns) not-significant by paired t-test (two-tailed); n=5 from 3 independent experiments.
5.3.8 Effect of the calcilytic NPS-2143 on salbutamol-induced $Ca^{2+}_i$ increase in BSMCs

In order to examine whether salbutamol enhances $Ca^{2+}_i$ concentration as a result of CaR activation, BSMCs were pretreated with NPS-2143 (1 µM) and then the cells were exposed to 2 mM $Ca^{2+}_o$-containing buffer followed by the addition of 1 µM salbutamol in the continued presence of the calcilytic. From the representative trace in Figure 5.10A, exposing the cells to NPS-2143 failed to inhibit the salbutamol-induced enhancement of $Ca^{2+}_i$ concentration. Quantification of the AUC of the global cell responses confirmed that the addition of the NPS-2143 failed to inhibit the salbutamol-induced increase in $Ca^{2+}_i$ concentration observed in the presence of 2 mM $Ca^{2+}_o$ (p>0.05) by unpaired two-tailed t-test. n=10-12 coverslips from 4 independent experiments.
Figure 5.10. Effect of the calcilytic NPS-2143 on salbutamol-enhanced Ca\textsuperscript{2+}\textsubscript{i} concentrations in BSMCs.

Panel A) Representative trace showing Ca\textsuperscript{2+}\textsubscript{i} changes (Fura-2 ratio) in 2 single cells (orange, blue) or “global” cluster of cells (black) in response to 2 mM Ca\textsuperscript{2+}o in presence or absence of salbutamol (1 µM) and continued presence of NPS-2143 (1 µM). Panel B) Quantification of changes in Ca\textsuperscript{2+}\textsubscript{i} concentrations as area under the curve/min. Data was normalised to the maximal response and presented as mean ± sem. (ns) not-significant by unpaired t test (two-tailed); n=10-12 from 4 independent experiments.
5.3.9 Effect of the calcilytic NPS-795 on salbutamol-induced $\text{Ca}^{2+}_\text{i}$ enhancement in BSMCs

As NPS-2143 failed to attenuate the salbutamol / 2 mM $\text{Ca}^{2+}_\text{o}$-induced increase in BSMC $\text{Ca}^{2+}_\text{i}$ concentration, another calcilytic was tested, namely NPS-795. Therefore, Fura-2 loaded cells were pretreated with NPS-795 (1 μM) then the cells were exposed to 2 mM $\text{Ca}^{2+}_\text{o}$-containing buffer followed by the addition of 1 μM salbutamol in the continued presence of the calcilytic. From the representative trace in Figure 5.11A, exposing the cells to the NPS-795 again failed to alter the salbutamol effect in enhancing $\text{Ca}^{2+}_\text{i}$ concentrations. Quantification of the AUC of the global cell responses confirmed that the addition of the NPS-795 failed to inhibit the salbutamol-induced $\text{Ca}^{2+}_\text{i}$ concentrations ($p>0.05$) by unpaired two-tailed t-test.
Figure 5.11. Effect of the calcilytic NPS-795 on salbutamol-enhanced Ca$^{2+}$\textsubscript{i} concentrations in BSMCs.

Panel A) Representative trace showing Ca$^{2+}$\textsubscript{i} changes (Fura-2 ratio) in 2 single cells (orange, blue) or "global" cluster of cells (black) in response to 2 mM Ca$^{2+}$\textsubscript{o} in presence or absence of salbutamol (1 µM) and continued presence of NPS-795 (1 µM). Panel B) Quantification of changes in Ca$^{2+}$\textsubscript{i} concentrations as area under the curve/min. Data was normalised to the maximal response and presented as mean ± sem. (ns) not-significant by unpaired t test (two-tailed); n=11-12 from 4 independent experiments.
5.3.10 Effect of salbutamol and NPS-2143 on the phosphorylation/activation of ERK, Akt and p38MAPK in BSMCs exposed to 2 mM Ca\(^{2+}\)\(_{o}\)

Having established that salbutamol enhances Ca\(^{2+}\)\(_{i}\) mobilisation in BSMCs and that calcilytics do not inhibit this response, it was necessary then to test the effect of salbutamol in the absence or presence of calcilytic on the phosphorylation of ERK, Akt and p38\(^{\text{MAPK}}\). Therefore, in this experiment, BSMCs were cultured in 35 mm dishes until reaching 80% confluence. Then, media was removed and cells were equilibrated in 0.5 mM Ca\(^{2+}\)\(_{o}\)-containing buffer for 20 minutes before treatment. Afterward, cells were incubated for 10 minutes at 37 °C in buffers containing 0.5 mM Ca\(^{2+}\)\(_{o}\) or 2 mM Ca\(^{2+}\)\(_{o}\) in the absence or presence of salbutamol (1 µM), NPS-2143 (1 µM) or both. Then, cells were lysed with RIPA-like buffer and the phosphorylation levels of the various proteins were determined by semi-quantitative immunoblotting. From the representative immunoblots in Figure 5.12A, in BSMCs salbutamol addition inhibited ERK phosphorylation but had no obvious effect on the phosphorylation levels of Akt and p38\(^{\text{MAPK}}\). Data quantification by densitometry confirmed that the salbutamol addition to 2 mM Ca\(^{2+}\)\(_{o}\)-containing buffer significantly inhibited ERK phosphorylation but did not significantly change the phosphorylation levels of Akt or p38\(^{\text{MAPK}}\) (Figure 5.12B). However, the calcilytic addition had no effect on the phosphorylation of any of the three proteins.
Figure 5.12. Effect of salbutamol and NPS-2143 on the phosphorylation levels of ERK, Akt and p38MAPK in BSMCs exposed to 2 mM Ca^{2+}o.

Panel A) Representative immunoblots showing the relative phosphorylation levels of ERK, Akt and p38MAPK in response to 2 mM Ca^{2+}o and salbutamol/calcilytic or both. β-actin was used as a loading control. Panel B) Quantification of changes in the phosphorylation levels of ERK, Akt and p38MAPK as % maximal response. (ns) not-significant; **P<0.01 and ***P<0.001 by repeated measures one-way ANOVA with Dunnett’s multiple comparisons post-test; n=8 from 4 independent experiments.
5.3.11 CaR expression in human bronchial smooth muscle cells

Finally, since the purpose of these experiments was to investigate potential CaR actions in BSMCs it was necessary to confirm the expression of CaR in human BSMCs lysate. For this, the mouse monoclonal ADD anti-CaR antibody (5C10, Invitrogen) was employed to detect CaR immunoreactivity. This antibody frequently detects two bands at ~140 (high mannose immature) and ~160 kDa (mature CaR) as seen in human CaR-HEK cells (Ward et al., 1998). First, BSMC lysate was processed for western blot analysis using the anti-CaR antibody, resulting in the detection of 3 immunoreactive bands at ~140, ~190 and another small-size band below 100 kDa (Figure 5.13A). Next, to assess whether any of these bands are likely to be CaR, CaR-HEK cells were used as a positive control to compare with BSMCs. The CaR-HEK and BSMCs lysates were diluted 4:1 with either reducing or non-reducing Laemmli buffer to show the CaR-specific bands in both reducing and non-reducing forms. Then, samples were processed for western blot analysis using the anti-CaR mouse monoclonal antibody. As expected, the CaR-HEK cells lysates revealed two bands of ~140 and ~160 kDa under reducing conditions that ran at twice the size under non-reducing conditions (Figure 5.13B). This confirms that the 140 and 160 kDa bands are most likely CaR proteins since under native conditions the CaR exists as a disulphide-linked homodimer (Ward et al., 1998). In contrast, in BSMCs lysates incubated with low primary antibody concentration (1:5000), two immunoreactive bands were detected under reducing conditions, corresponding to ~140 and ~190 kDa. However, under non-reducing conditions, these immunoreactive bands did not appear larger in size than when detected under reducing conditions. Moreover, when a BSMC immunoblot was probed with a higher primary antibody concentration (1:3000), more bands were observed but none that can be clearly ascribed to the CaR. On this basis no attempt was made to use siRNA knockdown to confirm CaR expression and as a result these experiments failed to positively identify CaR protein expression in BSMCs.
Figure 5.13. CaR expression in human BSMCs.

Panel A) Western blot analysis against CaR mouse monoclonal antibody (1:5000) on BSMCs lysate. Panel B) Western blot analysis against CaR antibody on both CaR-HEK cells (left) and BSMCs (right). β-met +/− indicates presence or absence of the reducing agent (β-mercaptoethanol) in the Laemmli buffer.
5.4 Discussion

5.4.1 PGE₂ induced cAMP production in CaR-HEK cells as determined by FRET ratiometry

Cyclic AMP is a second messenger which delivers extracellular signals from hormones such as adrenaline and glucagon into the cell through the activation of PKA and Epac (Patel and Gold, 2015). Over the last decade it has become possible to evaluate changes in intracellular cAMP levels using either PKA- or Epac-based real-time biosensors (Hamers et al., 2014). Thus, for the first time in our laboratory, I was able to employ Epac-based FRET ratiometry to detect changes in second messenger cAMP levels in living HEK-293 cells. This allowed investigation of the effect of CaR on cAMP modulation and to identify the upstream signals responsible for such changes. Particularly, the Epac (H187) is characterised by its increased affinity to cAMP as a result of a point mutation amino acid exchange (Q270E) in Epac structure (Storch et al., 2017) that reduces the disassociation constant of cAMP from 9.5 to 4 µM (Klarenbeek et al., 2015). The Epac sensor is a loss-of-FRET sensor in which raised cAMP levels resulted in increase the distance between the two fluorophores and a decrease in YFP/CFP ratio (Klarenbeek et al., 2011). However, in my results I instead reported the CFP/YFP ratio resulting in an increase in the trace amplitude reflecting the increased cAMP production. Also, it should be mentioned that for analysis we did not subtract the background for images and we did not correct for the CFP bleedthrough because we are interested in FRET kinetics rather than FRET efficiency. That is, it will not alter the relative FRET ratio amplitudes as all cells are treated in the same way.

These findings are in concordance with those of Klarenbeek et al. (2011) who reported that using TₐEpacVV, several cAMP generators including PGE₁, isoproterenol and forskolin plus IBMX successfully changed FRET ratio in HEK-293, mouse neuroblastoma (N1E-115) and human cervical cancer (HeLa) cells.
5.4.2 Effect of CaR activation on PGE$_2$-induced cAMP production in CaR-HEK cells transfected with the Epac biosensor

The measured changes in cAMP here reflect the balance between its $G_\alpha_s$-stimulated generation by AC, its degradation by PDEs and by the inhibitory effect of the PTx sensitive $G_\alpha_i$–mediated signalling pathway. It has been noted by Gerbino et al. (2005), that CaR activation may reverse agonist-dependent cAMP elevation by two mechanisms; i) direct stimulation of the $G_\alpha_i$ and/or ii) Ca$^{2+}_i$-dependent inhibitory effects on cAMP (Bruce et al., 2003; Brown and MacLeod, 2001). That is, raised Ca$^{2+}_i$ activates PDEs, predominantly from the PDE1 subfamily (Houslay and Milligan, 1997; Goraya et al., 2004), or activates the inhibitory isoforms of AC (Cooper, 2003). In this regard, de Jesus Ferreira et al. (1998) has reported that in the cortical thick ascending limb in kidney, CaR activation inhibits cAMP signalling via the activation of the inhibitory isoform of AC (type 6). Also, it has been reported that raised CaR-induced Ca$^{2+}_i$ mobilisation inhibits renin secretion from juxtaglomerular cells via activation of PDE and an inhibitory isoform of AC (Atchison and Beierwaltes, 2013).

Gerbino et al. (2005) demonstrated using a PKA-based FRET sensor for cAMP that CaR activation with the calcimimetics (NPS R-467) completely reversed the PGE$_2$-induced cAMP formation (determined by the elevation in the FRET ratio) in CaR-HEK cells. Also, Gerbino et al. (2005) has demonstrated that CaR activation with spermine prior to PGE$_2$ addition, completely inhibited the expected increase in FRET ratio induced by the PGE$_2$. Here, having demonstrated using the Epac biosensor that CaR activation with 5 mM Ca$^{2+}_o$ significantly inhibits PGE$_2$-induced cAMP accumulation (Figure 5.2) these data strongly support the findings of Gerbino et al. (2005). Moreover, having demonstrated that the addition of the calcimimetic R-568 to the buffer containing 3 mM Ca$^{2+}_o$ in the presence of PGE$_2$ significantly reversed the PGE$_2$-induced elevation in FRET ratio in HEK cells expressing CaR but not in wild-type HEK-293 cells (Figure 5.3) these data further support the idea that CaR activation terminates cAMP signalling.
5.4.3 Role of $G_{\alpha_{q/11}}$ mediated signalling in CaR-induced cAMP suppression

As mentioned earlier, stimulation of CaR causes $G_{\alpha_{q/11}}$/PLC activation resulting in IP$_3$ and DAG formation (Hannan et al., 2016). In turn, the IP$_3$ induces $Ca^{2+}_i$ mobilisation, which together with the DAG can activate a range of PKCs. The activated PKC, which we believe is most likely PKC$\alpha$ (Young et al., 2014; Bin-Khayat, 2016), then phosphorylates CaR$^{T888}$ in the ICD of CaR resulting in the inhibition of $Ca^{2+}_i$ mobilisation. Subsequent removal of this phosphate group by a protein phosphatase possibly PP2A (Davies et al., 2007) permits further $Ca^{2+}_i$ mobilisation again. Because of this sequence, addition of a PKC inhibitor such as GF109203X will prevent phosphorylation of CaR$^{T888}$ and thus enhance the CaR-induced $Ca^{2+}_i$ mobilisation as shown in (Figure 5.4) (Conigrave and Ward, 2013). Having optimised and validated the cAMP reporter in our system I then used it to investigate the effect of PKC inhibition on CaR-induced cAMP suppression. Indeed, GF109203X failed to increase the $Ca^{2+}_o$--induced suppression of cAMP (Figure 5.5) despite it enhancing CaR-induced $Ca^{2+}_i$ mobilisation as expected (Figure 5.4). Similarly, cotreatment with the $G_{\alpha_q}$ inhibitor YM-254890 failed to overcome the $Ca^{2+}_o$ inhibitory effect on cAMP further supporting the view that CaR does not mediate cAMP suppression through the activation of the $G_{\alpha_{q/11}}$ pathway. This reagent only became commercially available during the course of this project and thus it was interesting to see its lack of effect on cAMP levels. It should be noted that the concentration and formulation of YM-254890 used here was sufficient to abolish CaR-induced $Ca^{2+}_i$ mobilisation by a coworker in this laboratory (data not shown).

5.4.4 CaR-mediated cAMP suppression is PTx sensitive

Before the discovery of the FRET-based cAMP biosensors, several biochemical approaches were used to detect cAMP accumulation in the cells. The classical method for this involves prelabelling the cells with $^3$H-adenine followed by stimulation with the $G_{\alpha_s}$-activating agonist and then assay of $^3$H-adenine nucleotide in the cell homogenate (Salomon, 1991). However, to monitor $G_{\alpha_{i/o}}$ signalling-induced cAMP suppression it was necessary first to artificially increase the cAMP levels using forskolin or IBMX (Storch et al., 2017). This method is reliable but it
cannot be performed to measure the fluctuation in cAMP in single living cells. However, having such genetically-encoded biosensors allowed us to conduct lifetime single cell cAMP measurements. Indeed, it has been noted by Storch et al. (2017) that the cAMP sensor H187 in particular is sensitive to very small changes in cAMP and there may even be no need to prestimulate the cell with forskolin before examining the effect of $G_{ia}$-mediated cAMP decrease in single living cells. Though, in all our experiments I pretreated the cells with PGE$_2$ because I was interested in how CaR activation modulates cAMP generation. However, it might be useful to confirm that CaR activation inhibits cAMP production even in the absence of PGE$_2$.

Thus, using the H187 cAMP reporter I demonstrated that PTx was able to abolish the CaR inhibitory effect on cAMP production induced by PGE$_2$ (Figure 5.6), supporting the idea that CaR activation mediates cAMP suppression through the $G_{ia}$-dependent pathway, at least in CaR-HEK cells. Consistent with our finding, Gerbino et al. (2005) reported that in CaR-HEK cells pretreated with PTx, the response to PGE$_2$ was unaffected in the presence of spermine using a PKA-based FRET sensor. Similarly, it has been reported by Caroppo et al. (2004) that in gastric oxyntopeptic cells (OCs) expressing CaR, pretreatment with PTx inhibited the increase in pH induced by spermine. Indeed, it has been reported that in bovine PT cells, high Ca$_{2+o}$ concentrations decreased the cAMP accumulation enhanced by dopamine via activation of $G_{ia}$ mediated signalling thus leading to inhibition of PTH secretion (Chen et al., 1989). Also, it has been noted by Brown and MacLeod (2001) that PTx blocks the inhibition of PTH secretion mediated by high Ca$_{2+o}$ or other CaR agonists.

### 5.4.5 Effect of salbutamol and calcilytics on intracellular calcium mobilisation in BSMCs

It is well known that β-adrenergic agonists induce cAMP production via activation of the $G_{as}$-mediated intracellular signalling pathway (Figure 5.15) (Roozendaal, 2002; Xiang and Kobilka, 2003; Galaz-Montoya et al., 2017). However, it has been reported by Keller et al. (2014) that β$_2$-agonists increase Ca$_{2+1}$ concentrations inside cardiac myocytes mainly via store release from the ER, or, from outside the cell through store-operated channels. Pereira et al. (2017) similarly reported that β$_2$-agonists increase the release of Ca$_{2+1}$ from the sarcoplasmic reticulum (SR) via the
phosphorylation of the ryanodine receptor. Here, the demonstration that salbutamol enhances $\text{Ca}^{2+}_{\text{i}}$ mobilisation in BSMCs when $\text{Ca}^{2+}_{\text{o}}$ concentration is elevated supports the idea that $\beta$-adrenergic agonists can influence $\text{Ca}^{2+}_{\text{i}}$ mobilisation, possibly via PKA-dependent phosphorylation of IP$_3$R (Figure 5.15). However, in a very recent study, it has been reported that in HEK-293 cells, $\beta_2$AR activation causes robust $\text{Ca}^{2+}_{\text{i}}$ mobilisation via activation of the PLC pathway and binding of IP$_3$ to IP$_3$R present on the surface of ER and that this response is not mediated through any of $\text{G}_{\alpha_s}$, $\text{G}_{\alpha_i}$ or PKA (Galaz-Montoya et al., 2017). To further examine if $\beta_2$AR activation increased $\text{Ca}^{2+}_{\text{i}}$ concentration via activation of the PLC pathway in BSMCs, more experiments would need to be done, using for example the $\text{G}_{\alpha_{q11}}$ inhibitor or a FRET-based $\text{G}_{\alpha_q}$ activity sensor.

Further, it should be noted that neither calcilytic, NPS-2143 or NPS-795, could overcome the effect of salbutamol / raised $\text{Ca}^{2+}_{\text{o}}$ concentration on $\text{Ca}^{2+}_{\text{i}}$ mobilisation, suggesting that this effect is not mediated through the activation of CaR and it is most probably a result of a direct effect of $\beta_2$AR activation though only in the presence of raised $\text{Ca}^{2+}_{\text{o}}$ concentration. Since the salbutamol was without effect on $\text{Ca}^{2+}_{\text{i}}$ mobilisation when the $\text{Ca}^{2+}_{\text{o}}$ concentration was only 0.5 mM (Figure 5.9), then it seems likely that the salbutamol could elicit $\text{Ca}^{2+}_{\text{o}}$ influx from outside the cell provided that the $\text{Ca}^{2+}_{\text{o}}$ concentration was sufficiently high (Figure 5.15).
CaR activation could stimulate $G_{\alpha_q}/PLC$ or $G_{\alpha_i}$-mediated cAMP suppression pathways. PLC activation increases the production of IP$_3$ which then binds to IP$_3$R present on the surface of ER and thus increases the release of Ca$^{2+}$ to the cytoplasm. β-adrenergic receptor activation increases cAMP via $G_{\alpha_s}$-mediated intracellular signalling pathway. cAMP could increase Ca$^{2+}$ concentration via PKA-dependent phosphorylation of IP$_3$ receptor or enhancing Ca$^{2+}$ influx from outside the cell.

5.4.6 Effect of salbutamol and calcilytics on activation of ERK, Akt and p38$^{MAPK}$ in BSMCs

It was shown that addition of the β$_2$-adrenergic agonist salbutamol decreased ERK phosphorylation significantly in BSMCs (Figure 5.12). This finding is consistent with the finding of a recent study Keränen et al. (2017) which reported that salbutamol inhibits inflammatory gene expression through cAMP-dependent ERK inhibition in macrophages. Similarly, it has been reported by Hu et al. (2012) that salbutamol reduced the inflammation induced by lipopolysaccharide (LPS) by inhibiting ERK phosphorylation in bone marrow-derived dendritic cells. Also, Fogli et al. (2013) reported that salbutamol, along with PPAR agonist, promoted the G1/S phase of the cell cycle in human bronchial smooth muscle cells via ERK inhibition.

On the other hand, it has been reported by Yarova et al. (2015) that calcilytics inhibit ERK, p38$^{MAPK}$ and Akt phosphorylation induced by CaR activation in airway smooth muscle (ASM) cells. However, the current results demonstrated that
calcilytics have no effect on the phosphorylation level of ERK, p38MAPK and Akt in BSMCs (Figure 5.12). Thus, the calcilytic neither enhanced nor antagonised the salbutamol effect on either Ca\textsuperscript{2+}i mobilisation or ERK activation in BSMCs. This may be because BSMCs lack sufficient CaR protein, or, it may need to be done on cells derived from asthmatic patients in which the CaR expression levels may have been increased. Regarding the former, CaR downregulation in primary cell culture is a common problem and means there are very few primary cell models of CaR signalling.

Also, it should be noted that one of our objectives was to use the FRET-based Epac biosensor to test the effects of salbutamol and the calcilytics on cAMP accumulation in the BSMCs. However, it was not possible to achieve sufficient expression of the sensor in the cells except on a single occasion but where the salbutamol failed to alter the FRET signal (not shown) and thus no further experiments were performed on this. It should be noted that use of BSMCs should be limited to less than four passages as loss of the cellular phenotype may be observed after that (Yarova et al., 2015).

5.5 **Summary**

The data presented in this chapter demonstrated that G\textalpha\textsb{s}-agonist stimulates cAMP activation and that CaR activation reversed this increase via activation of G\textalpha\textsb{i}-dependent pathway. In BSMCs, the \beta\textsb{2}AR agonist (salbutamol) increased Ca\textsuperscript{2+}i mobilisation and inhibited ERK phosphorylation but that this did not appear to be CaR-dependent in this particular cell-type.
CHAPTER 6

General Discussion and Conclusion
6.1 General discussion

The parathyroid gland’s principal function is to maintain Ca\(^{2+}\) homeostasis via the action of parathyroid hormone which its secretion is under the control of CaR acting through the Ga\(_{q/11}\) and (possibly) Ga\(_i/o\) intracellular signalling pathway(s). However, the underlying cellular driving force for tonic PTH secretion is poorly understood especially given that PTH lacks the secretion-stimulating signals that most other endocrine glands possess. It is believed that agonists that generate cAMP via activation of Ga\(_s\)-coupled GPCRs may increase PTH secretion (Brown et al., 1977a; Brown et al., 1977b; Gardner et al., 1978; Shoback and Brown, 1984; Brown and Swartz, 1985). Here our hypothesis is that PTG endogenously secretes Ga\(_s\)-coupled GPCR agonists such as prostaglandins and histamine, which act in an autocrine/paracrine fashion to stimulate cAMP formation and thus elicit PTH secretion (Figure 6.1). In this project, I performed the molecular experiments to identify the expression of some Ga\(_s\)-coupled GPCRs and their ligand synthases particularly in bovine PTG and to investigate the signalling crosstalk between Ga\(_s\)-coupled receptors and the Ga\(_q\)-coupled CaR in CaR-HEK cells which shares various characteristics with PTG cells making it, for now, the best cell model to use.

![Figure 6.1. Hypothetical model of how endogenous prostanoids may elicit autocrine / paracrine stimulation of EP\(_4\) and prostacyclin receptors.](image)

In PT cell \(A\), arachidonic acid is converted to prostanoid (PGE\(_2\) and PGI\(_2\)) via COX1/2. PGE\(_2\) and PGI\(_2\) act on adjacent PT cell \(B\) via Ga\(_s\)-linked receptors (EP\(_4\)R and IPR respectively) to increase intracellular cAMP levels and thus drive PTH secretion.
Analysing gene microarray data performed on cDNA obtained from bovine and murine PTG, suggests the probable expression of some Ga\(_\alpha\)-coupled GPCRs such as EP\(_4\)R, EP\(_2\)R, histamine receptor, \(\beta_2\) adrenergic receptor and Calcitonin receptor family. And in order to understand the role of cAMP generation in PTG, here first I confirmed that gene and protein expression of the EP\(_4\)R and the gene expression of its ligand synthase in bovine PTG. These findings are consistent with the functional data from the lab of our collaborator Professor Arthur Conigrave (University of Sydney) who demonstrated that cotreatment with inhibitors of either COX-2 or EP\(_4\)R acutely and reversibly suppressed the intrinsic release of PTH from human perfused parathyroid cells (Szczawinska et al., 2015). Also, in support of the data obtained from the gene microarray analysis performed on the cDNA of bovine and murine PTG, I have confirmed the gene and protein expression of CALCRL as well as the gene expression of its associated components including RAMP-1, RAMP-2, RAMP-3 and adrenomedullin. The importance of RAMP-1 and RAMP-3 might be explained by their role in introducing CaR to the cell surface (Bouschet et al., 2005; Desai et al., 2014; Hay and Pioszak, 2016). Furthermore, the mRNA expression of the CALCRL and its ligand CGRP \(\alpha\) and \(\beta\) were apparently \(\text{Ca}^{2+}\text{O}\)-sensitive similar to the other known calciotropic genes; CaR, PTH and VDR according to the murine microarrays which comparing the effect of \(\text{Ca}^{2+}\text{O}\) concentrations on the level of genes expression (Table 3.3). While this remains to be fully proven, it does raise the question of what functional role in PT physiology CALCRL signalling may play as this has not been previously investigated. Together, these molecular observations support the idea that the PT gland could maintain the spontaneous secretion of PTH via an intrinsic mechanism.

There is some evidence that increasing cAMP levels such as forskolin could enhance CaR-mediated \(\text{Ca}^{2+}\text{i}\) mobilisation at least in CaR-HEK cells (Gerbino et al., 2005; Campion, 2013). In support to this observation, I have confirmed that cAMP-generating agonists including PGE\(_2\), isoprenaline and histamine enhanced CaR-induced \(\text{Ca}^{2+}\text{i}\) mobilisation at subthreshold \(\text{Ca}^{2+}\text{O}\) concentrations and that this effect was significantly inhibited with calcilytic. Also, it has been demonstrated that PGE\(_2\) and isoprenaline lowered the EC\(_{50}\) for \(\text{Ca}^{2+}\text{O}\)-induced \(\text{Ca}^{2+}\text{i}\) mobilisation. However, it is still unclear how increasing cAMP enhances CaR-induced \(\text{Ca}^{2+}\text{i}\) mobilisation. Cyclic AMP might influence \(\text{Ca}^{2+}\text{i}\) concentration via PKA-dependent
phosphorylation of either the IP₃ receptor or plasma membrane Ca²⁺-ATPase (PMCA) responsible for Ca²⁺ clearance (Bruce et al., 2003). Alternatively, the cAMP generation may lead to PKA-mediated (Manni et al., 2008; Petersen et al., 2008) phosphorylation of one or both of the CaR’s putative PKA sites S-899 and S-900 (Garrett et al., 1995; Bai, 2004; Stepanchick et al., 2010). However, unpublished data from our laboratory suggests that neither PKA inhibition, nor mutation of these two PKA sites can affect CaR-induced Ca²⁺ᵢ mobilisation (Campion, 2013). But, whatever the mechanism, stimulation of Gαs-coupled receptors increase cAMP levels sufficiently to potentiate CaR activity. If this were also true in PT cells, then by then lowering cAMP levels (via activation of Gαᵢ/o or a Ca²⁺ᵢ-calmodulin dependent PDE) this could represent a (Figure 4.20A) short-loop inhibition to dampen excess PTH secretion before it had time to take effect i.e. to raise serum calcium concentrations (long-loop feedback). The presence of such short-loop feedback might even provide a good explanation for the normal ultradian pulsatile pattern of PTH secretion (reviewed in (Chiavistelli et al., 2015)). But in any case, this interaction needs first to be demonstrated in PT cells and not just in CaR-HEK cells.

It was first reported by Kifor et al. (2001) that in both bovine PTG and CaR-HEK cells, high Ca²⁺ᵀ induces ERK1/2 phosphorylation. Also, unpublished data from our laboratory has shown that Ca²⁺ᵀ-mediated CaR activation enhances the phosphorylation of other kinases including IGFR, IκBα and P70 S6 kinase (Bin-Khayat, 2016). Moreover, there is evidence that increasing cAMP level with forskolin enhances CaR-induced ERK1/2 activation in CaR-HEK cells (Campion, 2013). Here I have demonstrated that PGE₂ significantly enhanced the CaR-mediated phosphorylation of ERK1/2 but tended to inhibit P70 S6 kinase in CaR-HEK cells exposed to moderate Ca²⁺ᵀ concentrations (Figure 4.15). PGE₂ specifically mediated ERK1/2 phosphorylation via activation of EP₄R as this effect was inhibited with the selective EP₄R antagonist L161982 (**p<0.01). I have also demonstrated that cotreatment with three Gαᵣ-agonists including PGE₂, isoprenaline and histamine at subthreshold concentrations significantly enhanced CaR-induced Ca²⁺ᵢ mobilisation in the presence of relatively low Ca²⁺ᵀ concentration (2 mM) whereas each was without effect on its own. These observations further raise the possibility that CaR signalling could be modulated by modest physiological stimuli acting through a
variety of Go-s-coupled GPCRs. However, these signalling observations will also need to be confirmed in cells that express the CaR endogenously, specifically parathyroid cells.

CaR activation suppresses cAMP accumulation in PTG and CaR-HEK cells (Brown and MacLeod, 2001) and this might occur via pertussis toxin-sensitive Gaio-mediated signalling (Chen et al., 1989; Gerbino et al., 2005). However, there is also evidence that in PT, CaR could suppress cAMP via Ga,i-independent activation of phosphodiesterase (PDE) isoform-1 which degrades cAMP in response to elevated Ca2+ levels (Conigrave and Ward, 2013). Here, using a FRET based Epac-sensor I have demonstrated that PGE2 does stimulate cAMP accumulation in CaR-HEK cells while high Ca2+ or R568-mediated CaR activation lowers cAMP levels via a pertussis toxin-sensitive signalling pathway. Furthermore, the CaR-induced decrease in intracellular cAMP levels was not altered by GF102903X-induced PKC inhibition, despite this intervention being known to increase CaR-induced Ca2+ mobilisation (Davies et al., 2007; McCormick et al., 2010).

Finally, β2AR agonists such as salbutamol represent the first line of asthma treatment as they relax and reverse the bronchoconstriction occurring during an acute attack and this occurs via activation of Go-s-mediated intracellular signalling (Johnson, 1998; Johnson and Rennard, 2001; Burgel et al., 2017). Recently, it has been suggested that calcilytics, beside their role in treating mineral disease, may also represent a potential therapy in treating asthma by antagonising the effect of the cationic compounds-mediated CaR activation in airway smooth muscle cells (Yarova et al., 2015). Therefore, here I first showed that increasing Ca2+ concentrations in human bronchial smooth muscle cells (BSMCs) does permit the β2AR agonist salbutamol to increase Ca2+ mobilisation and inhibit ERK1/2 phosphorylation whereas it is without effect under conditions of low Ca2+ concentrations. However, neither effect could be inhibited by calcilytic cotreatment suggesting that the Ca2+ effect does not occur via CaR activation in BSMCs, at least in these BSMCs derived from a non-asthmatic donor. Thus, it would be interesting to repeat this in future in cells of asthmatic origin.
6.2 General conclusion

The data presented in this project increases our understanding of the crosstalk between CaR and Ga\textsubscript{s}-coupled receptors. That is, with modest concentrations of cAMP-generating agonists enhancing CaR-induced Ca\textsuperscript{2+}\textsubscript{i} mobilisation and CaR stimulation attenuating enhanced cAMP levels. This may be of greatest relevance in vivo where endogenous agonists of Ga\textsubscript{s}-coupled GPCRs may be more abundant than in the simple salt solutions used in vitro.

Understanding how cAMP and CaR signalling are integrated will help us to determine the mechanism of PTH secretion as well as the CaR-mediated regulation of secretion. With the increasing incidence of osteoporosis and kidney disease these mechanisms are not only important to understand the physiology of PTH secretion but to identify novel therapeutics in treatment of diseases associated with mineral dysfunction.
REFERENCES


Bin-Khayat, M. E. (2016) Protein kinase involvement in wild-type and mutant calcium-sensing receptor signalling. PhD, University of Manchester.


186


Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. & Lefkowitz, R. (1992) Constitutive activation of the alpha 1B-adrenergic receptor by all amino acid
substitutions at a single site. Evidence for a region which constrains receptor activation. *Journal of Biological Chemistry*, 267(3), 1430-1433.


Structures and Computational Simulations. *Journal of Medicinal Chemistry*, “ePub”.


195


Appendix

Part of the data in this thesis was published as an abstract.


Part of the data in this thesis was presented at national and international conferences.


Albar, H., Conigrave, A. D. & Ward, D. T. (May 11th - 13th, 2017) Signal Crosstalk between CaSR and Gs-coupled receptors. Poster session presented at The 3rd International Symposium on The Calcium-Sensing Receptor (CaSR). Florence, Italy. (The abstract for this poster was awarded one of the ASBMR Young Investigator Awards).