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Sites of interaction between calcitonin-family peptides and their receptors

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

The University of Auckland, 2011
Abstract

The calcitonin-family of peptides comprises calcitonin, amylin, calcitonin gene-related peptides (CGRPs), adrenomedullin (AM) and AM2. Their receptors are calcitonin receptor-like receptor (CLR) or calcitonin receptor (CTR) and receptor activity-modifying protein (RAMP) complexes. RAMP1 with CLR constitutes the CGRP receptor whereas RAMP2 or 3 with CLR generates the AM receptors. Amylin receptors are formed from CTR interactions with RAMPs. The association of RAMPs with CLR or CTR can alter receptor trafficking, pharmacology or signalling capabilities. Despite the broad pharmacological potential as drug targets for treatments of diseases such as diabetes, migraine and osteoporosis, the calcitonin peptide family receptors are not fully characterised. This is mainly due to the difficulty in the structural determination and the complexity of the interaction system. To generate drugs that target these receptors, a clearer understanding of the role of each receptor component in peptide binding is needed.

In the thesis, structure-function relationships of residues or regions in the extracellular N termini of RAMPs were determined aiming to identify residues or regions that are important for ligand to receptor interactions for the calcitonin family peptides. The data generated have emphasised the importance of helices 2 and 3 of RAMP to peptide to receptor interactions. In particular, E74 of RAMP3 has been demonstrated to be crucial for high affinity binding and high potency response of AM at the AM2 receptor. In addition, a naturally occurring variant of the human CTR lacking the N-terminal 47 amino acids has been characterised in this thesis. For the first time, it has been shown that this truncated variant is still able to form a functional amylin receptor with RAMP1 at the cell surface. The observation of reduced potency for some peptide ligands at the truncated CTR suggests that the truncated region may contain some residues that are involved in the interactions for these peptides. In summary, the findings in this thesis have contributed to our understanding of the ligand to receptor interactions for the calcitonin family peptides. Residues and regions identified in this thesis could potentially provide some targeting points for future investigations for rational drug design.
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# Table of Contents

Abstract ................................................................................................................................. i
Acknowledgement .................................................................................................................. ii
Publications Arising From This Thesis ................................................................................ iii
Publications from thesis to date: ............................................................................................ iii
Publications in preparation: ..................................................................................................... iii
Table of Contents ................................................................................................................... iv
List of Figures .......................................................................................................................... xi
List of Tables .......................................................................................................................... xvi

## Chapter 1 Introduction ....................................................................................................... 1

1.1 Overview .......................................................................................................................... 1

1.1.1 The calcitonin family of peptides ................................................................................. 1
1.1.2 The receptors of the calcitonin family peptides .......................................................... 1

1.2 CGRP ............................................................................................................................... 3
1.3 AM ................................................................................................................................... 5
1.4 CT .................................................................................................................................... 6
1.5 Amy ..................................................................................................................................... 7

1.6 CLR/RAMPs receptor complexes (CGRP and AM receptors) ..................................... 7

1.6.1 Pharmacology of CGRP receptors ................................................................................. 7
1.6.2 AM receptors ................................................................................................................ 9
1.6.3 Intracellular signalling (CGRP and AM receptors) ..................................................... 10

1.7 CTR and CTR/RAMP receptor complexes (CT and Amy receptors) ......................... 11

1.7.1 CTR ............................................................................................................................. 11

1.7.1.1 Receptor heterogeneity ......................................................................................... 12
1.7.1.2 Receptor pharmacology ....................................................................................... 13

1.7.2 Amy receptors ............................................................................................................ 14

1.7.2.1 Receptor heterogeneity ......................................................................................... 14
1.7.2.2 Receptor pharmacology ....................................................................................... 14

1.7.3 Intracellular signalling (CTR and Amy receptors) ................................................... 15

1.8 Ligand binding to the calcitonin peptide family receptors ............................................. 17

1.8.1 GPCRs ......................................................................................................................... 17
1.8.2 Mechanisms of peptide interaction with family B GPCRs ........................................ 18
1.8.3 Structure of family B GPCRs and their ligands ......................................................... 19
1.8.4 Residues of CLR and CTR important for ligand to receptor interactions .......... 21

1.9 RAMP family of proteins ............................................................................................... 23

1.9.1 Other receptors interacting with RAMPs .................................................................. 23
Chapter 2

2.1 Materials

2.1.1 Peptides

2.1.2 Reagents

2.1.3 Inorganic solutions

2.1.4 Antibodies

2.1.5 Cell culture reagents

2.1.6 Transfection reagents

2.1.7 DNA constructs

2.1.7.1 RAMP constructs

2.1.7.2 CLR constructs

2.1.7.3 CTR constructs

2.1.8 Mammalian cells

2.2 Methods

2.2.1 Site-directed mutagenesis

2.2.1.1 Primer Design

2.2.1.2 Mutagenesis

2.2.1.3 Transformation

2.2.2 Site-directed mutagenesis

2.2.2.1 Primer Design

2.2.2.2 Mutagenesis

2.2.2.3 Transformation

2.2.3 Site-directed mutagenesis

2.2.3.1 Primer Design

2.2.3.2 Mutagenesis

2.2.3.3 Transformation

2.2.4 Site-directed mutagenesis

2.2.4.1 Primer Design

2.2.4.2 Mutagenesis

2.2.4.3 Transformation

2.2.5 Site-directed mutagenesis

2.2.5.1 Primer Design

2.2.5.2 Mutagenesis

2.2.5.3 Transformation
Chapter 3 Pharmacological characterisation of RAMP1/3 chimaeras in AMY$_{1(a)}$ receptors

3.1 Introduction ........................................................................................................... 69
3.2 Results .................................................................................................................... 72
  3.2.1 Effects of RAMP1/3 chimaeras on receptor expression .................................... 72
    3.2.1.1 Cell-surface expression ........................................................................... 72
    3.2.1.2 Total expression of selected RAMP1/3 chimaeric constructs ............... 73
  3.2.2 Functional characterisation using cAMP assays .............................................. 75
    3.2.2.1 RAMP$_{318-21}$ AMY$_{1(a)}$ ................................................................. 76
    3.2.2.2 RAMP$_{322-25}$ AMY$_{1(a)}$ ................................................................. 77
    3.2.2.3 RAMP$_{326-29}$ AMY$_{1(a)}$ ................................................................. 78
    3.2.2.4 RAMP$_{330-33}$ AMY$_{1(a)}$ ................................................................. 79
    3.2.2.5 RAMP$_{334-37}$ AMY$_{1(a)}$ ................................................................. 80
    3.2.2.6 RAMP$_{338-41}$ AMY$_{1(a)}$ ................................................................. 81
    3.2.2.7 RAMP$_{342-45}$ AMY$_{1(a)}$ ................................................................. 82
    3.2.2.8 RAMP$_{346-49}$ AMY$_{1(a)}$ ................................................................. 83
    3.2.2.9 RAMP$_{350-53}$ AMY$_{1(a)}$ ................................................................. 84
    3.2.2.10 RAMP$_{354-57}$ AMY$_{1(a)}$ ............................................................... 85
    3.2.2.11 RAMP$_{358-61}$ AMY$_{1(a)}$ ............................................................... 86
    3.2.2.12 RAMP$_{362-65}$ AMY$_{1(a)}$ ............................................................... 87

Table of Contents

  2.2.1.4 Isolation of plasmid for sequencing .......................................................... 56
  2.2.2 Maxiprep DNA preparations ................................................................. 57
    2.2.2.1 Maxiprep DNA preparations from SDM products .............................. 57
    2.2.2.2 Maxiprep DNA preparations from existing maxiprep DNAs .............. 57
  2.2.3 Cell culture .................................................................................................... 58
  2.2.4 Transient transfection ................................................................................... 58
  2.2.5 Cell-based ELISA ......................................................................................... 59
    2.2.5.1 Cell-surface expression measured by mycRAMP1 vs HA-CLR ........... 60
    2.2.5.2 Determination of anti-CTR antibody (9B4) specificity .................... 61
  2.2.6 Radio-receptor cAMP assay .......................................................................... 63
    2.2.6.1 Stimulation of cells ............................................................................. 63
    2.2.6.2 cAMP measurement .......................................................................... 64
  2.2.7 Membrane preparation .................................................................................. 64
  2.2.8 Cell lysate preparation .................................................................................. 65
  2.2.9 Protein content assay ................................................................................... 65
  2.2.10 $^{125}$I-hAM$_{13-52}$ binding assay .............................................................. 66
  2.2.11 Western Blotting ......................................................................................... 66
  2.2.12 Data analysis and statistical procedures .................................................... 67
Chapter 4 Characterisation of the effect of incorporating RAMP3 residues into RAMP1 on CGRP and AMY1(a) receptor function

4.1 Introduction ......................................................... 108
4.2 Results .................................................................. 111
  4.2.1 mycRAMP1 mutants with HA-CLR; CGRP receptor .......... 111
    4.2.1.1 Cell-surface expression ........................................ 111
    4.2.1.2 Functional characterisation using cAMP assays .......... 112
      4.2.1.2.1 A34E CGRP receptor .................................... 113
      4.2.1.2.2 V46D CGRP receptor .................................... 114
      4.2.1.2.3 W74E CGRP receptor .................................... 115
      4.2.1.2.4 A87P CGRP receptor .................................... 116
      4.2.1.2.5 E88L CGRP receptor .................................... 117
      4.2.1.2.6 V89A CGRP receptor .................................... 118
      4.2.1.2.7 F93I CGRP receptor .................................... 119
      4.2.1.2.8 S103N CGRP receptor .................................... 120
      4.2.1.2.9 Characterisation of triple mutant RAMP386-89 CGRP receptor ..... 121
  4.2.2 mycRAMP1 mutants with HA-CT(a); AMY1(a) receptor ...... 123
    4.2.2.1 Cell-surface expression ........................................ 123
    4.2.2.2 Functional characterisation using cAMP assays .......... 124
      4.2.2.2.1 A34E AMY1(a) receptor .................................. 124
      4.2.2.2.2 V46D AMY1(a) receptor .................................. 125
5.2 Results

5.2.1 RAMP3 mutants with HA-CLR; AM2 receptor

5.2.1.1 Cell-surface expression

5.2.1.2 Functional characterisation using cAMP assays

5.2.1.2.1 E35A AM2 receptor

5.2.1.2.2 D46V AM2 receptor

5.2.1.2.3 E74W AM2 receptor

5.2.1.2.4 P87A AM2 receptor

5.2.1.2.5 L88E AM2 receptor

5.2.1.2.6 A87P AM2 receptor

5.2.1.2.7 I93F AM2 receptor

5.2.1.2.8 N103S AM2 receptor

5.2.1.3 Characterisation of RAMP1_{86-89} AM2 receptor

5.2.2 RAMP3 mutants with HA-CT_{(a)}; AM3(a) receptor

5.2.2.1 E35A AM3(a) receptor

5.2.2.2 D46V AM3(a) receptor

5.2.2.3 E74W AM3(a) receptor

5.2.2.4 P87A AM3(a) receptor

5.2.2.5 L88E AM3(a) receptor

5.2.2.6 A89V AM3(a) receptor

5.2.2.7 I93F AM3(a) receptor

5.2.2.8 N103S AM3(a) receptor

5.3 Discussion

Chapter 5 Characterisation of the effect of incorporating individual RAMP1 residues into RAMP3 on AM2 and AM3(a) receptor function

5.1 Introduction

5.2 Results

5.2.1 RAMP3 mutants with HA-CLR; AM2 receptor

5.2.1.1 Cell-surface expression

5.2.1.2 Functional characterisation using cAMP assays

5.2.1.2.1 E35A AM2 receptor

5.2.1.2.2 D46V AM2 receptor

5.2.1.2.3 E74W AM2 receptor

5.2.1.2.4 P87A AM2 receptor

5.2.1.2.5 L88E AM2 receptor

5.2.1.2.6 A87P AM2 receptor

5.2.1.2.7 I93F AM2 receptor

5.2.1.2.8 N103S AM2 receptor

5.2.1.3 Characterisation of RAMP1_{86-89} AM2 receptor

5.2.2 RAMP3 mutants with HA-CT_{(a)}; AM3(a) receptor

5.2.2.1 E35A AM3(a) receptor

5.2.2.2 D46V AM3(a) receptor

5.2.2.3 E74W AM3(a) receptor

5.2.2.4 P87A AM3(a) receptor

5.2.2.5 L88E AM3(a) receptor

5.2.2.6 A89V AM3(a) receptor

5.2.2.7 I93F AM3(a) receptor

5.2.2.8 N103S AM3(a) receptor

5.3 Discussion

Chapter 6 Structure-function relationships of residues at position 74 of RAMP1 and RAMP3 in CGRP and AM2 receptors

6.1 Introduction

6.2 Results
Table of Contents

7.4.1.4 hαCGRP response ................................................................. 193
7.4.1.5 hβCGRP response ................................................................. 194
7.4.1.6 TyrαhCGRP response ............................................................. 195
7.4.2 Antagonist pharmacology ......................................................... 196
  7.4.2.1 sCT<sub>8-32</sub> antagonism ..................................................... 196
  7.4.2.2 AC187 antagonism ............................................................... 198
7.5 Role of I347T mutation ............................................................... 200
  7.5.1 Cell-surface expression ............................................................ 200
  7.5.2 cAMP assay ........................................................................... 202
7.6 Discussion .................................................................................... 203

Chapter 8 Discussion and Conclusion ............................................. 209
  8.1 Overview ..................................................................................... 209
  8.2 Mutagenesis study in RAMPs 1 and 3 ........................................... 209
    8.2.1 RAMP1/3 chimaera study ....................................................... 210
    8.2.2 Evaluation of chimaera approach ........................................... 214
    8.2.3 Strategic substitutions in RAMPs 1 and 3 ............................... 215
      8.2.3.1 RAMPs 1 and 3 mutants with CLR .................................... 215
      8.2.3.2 RAMPs 1 and 3 mutants with CT<sub>(a)</sub> ............................ 222
  8.3 Δ(1-47)hCT<sub>(a)</sub> characterisation .............................................. 223
  8.4 Conclusion and future work ......................................................... 225
Appendix ................................................................................................ A1
References ............................................................................................. R1
List of Figures

Figure 1.1 Amino acid sequence alignment of the calcitonin family of peptides .......... 1
Figure 1.2 Apparent pA₂ estimates of CGRP₁₈₋₃₇ on human cell lines and tissues .......... 9
Figure 1.3 A schematic diagram showing a common structure shared by family B GPCRs .................................................................................................................. 18
Figure 1.4 Amino acid sequence alignment of three human RAMPs ......................... 25
Figure 1.5 The crystal structure of the extracellular N-terminal domain of human RAMP1 and the human RAMP3 N-terminal domain model ........................................ 26
Figure 2.1 Cell-surface expression of the CGRP receptors containing mycRAMP1 mutants W74E, W74Y, W74A, W74N and WT in complex with HA-CLR ....................... 61
Figure 2.2 Cell-surface expression of CTₐ constructs and CLR measured using 9B4 ... 62
Figure 2.3 Comparison of cell-surface expression of HA-CTₐ constructs measured using 9B4 and anti-HA antibody .................................................................................. 63
Figure 3.1 Amino acid sequence alignment of human RAMPs 1 and 3 ...................... 71
Figure 3.2 ELISA data for RAMP1/3 chimaeras expressed with HA-CLR ............... 73
Figure 3.3 Effects of three RAMP1/3 chimaeras on total cellular expression of mycRAMP1 in complex with HA-CLR .................................................................................. 74
Figure 3.4 cAMP data generated at WT AMY₁(a) and AMY₃(a) receptors .............. 75
Figure 3.5 cAMP data generated at RAMP₃₁₈₋₂₁ AMY₁(a) and WT AMY₁(a) receptors .. 76
Figure 3.6 cAMP data generated at RAMP₃₂₂₋₂₅ AMY₁(a) and WT AMY₁(a) receptors .. 77
Figure 3.7 cAMP data generated at RAMP₃₂₆₋₂₉ AMY₁(a) and WT AMY₁(a) receptors . 78
Figure 3.8 cAMP data generated at RAMP₃₃₀₋₃₃ AMY₁(a) and WT AMY₁(a) receptors .. 79
Figure 3.9 cAMP data generated at RAMP₃₃₄₋₃₇ AMY₁(a) and WT AMY₁(a) receptors .. 80
Figure 3.10 cAMP data generated at RAMP₃₃₈₋₄₁ AMY₁(a) and WT AMY₁(a) receptors 81
Figure 3.11 cAMP data generated at RAMP₃₄₂₋₄₅ AMY₁(a) and WT AMY₁(a) receptors 82
Figure 3.12 cAMP data generated at RAMP₃₄₆₋₄₉ AMY₁(a) and WT AMY₁(a) receptors 83
Figure 3.13 cAMP data generated at RAMP₃₅₀₋₅₃ AMY₁(a) and WT AMY₁(a) receptors 84
Figure 3.14 cAMP data generated at RAMP₃₅₄₋₅₇ AMY₁(a) and WT AMY₁(a) receptors 85
Figure 3.15 cAMP data generated at RAMP₃₅₈₋₆₁ AMY₁(a) and WT AMY₁(a) receptors 86
Figure 3.16 cAMP data generated at RAMP₃₆₂₋₆₅ AMY₁(a) and WT AMY₁(a) receptors 87
Figure 3.17 cAMP data generated at RAMP₃₆₆₋₆₉ AMY₁(a) and WT AMY₁(a) receptors 88
Figure 3.18 cAMP data generated at RAMP₃₇₀₋₇₃ AMY₁(a) and WT AMY₁(a) receptors 89
Figure 3.19 cAMP data generated at RAMP₃₇₄₋₇₇ AMY₁(a) and WT AMY₁(a) receptors 90
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.20</td>
<td>cAMP data generated at RAMP3$<em>{78-81}$ AMY$</em>{(a)}$ and WT AMY$_{(a)}$ receptors</td>
<td>91</td>
</tr>
<tr>
<td>3.21</td>
<td>cAMP data generated at RAMP3$<em>{82-85}$ AMY$</em>{(a)}$ and WT AMY$_{(a)}$ receptors</td>
<td>92</td>
</tr>
<tr>
<td>3.22</td>
<td>cAMP data generated at RAMP3$<em>{86-89}$ AMY$</em>{(a)}$ and WT AMY$_{(a)}$ receptors</td>
<td>93</td>
</tr>
<tr>
<td>3.23</td>
<td>cAMP data generated at RAMP3$<em>{90-93}$ AMY$</em>{(a)}$ and WT AMY$_{(a)}$ receptors</td>
<td>94</td>
</tr>
<tr>
<td>3.24</td>
<td>cAMP data generated at RAMP3$<em>{94-97}$ AMY$</em>{(a)}$ and WT AMY$_{(a)}$ receptors</td>
<td>95</td>
</tr>
<tr>
<td>3.25</td>
<td>cAMP data generated at RAMP3$<em>{98-101}$ AMY$</em>{(a)}$ and WT AMY$_{(a)}$ receptors</td>
<td>96</td>
</tr>
<tr>
<td>3.26</td>
<td>cAMP data generated at RAMP3$<em>{102-105}$ AMY$</em>{(a)}$ and WT AMY$_{(a)}$ receptors</td>
<td>97</td>
</tr>
<tr>
<td>3.27</td>
<td>cAMP data generated at RAMP3$<em>{106-109}$ AMY$</em>{(a)}$ and WT AMY$_{(a)}$ receptors</td>
<td>98</td>
</tr>
<tr>
<td>3.28</td>
<td>cAMP data generated at RAMP3$<em>{110-113}$ AMY$</em>{(a)}$ and WT AMY$_{(a)}$ receptors</td>
<td>99</td>
</tr>
<tr>
<td>3.29</td>
<td>cAMP data generated at RAMP3$<em>{114-117}$ AMY$</em>{(a)}$ and WT AMY$_{(a)}$ receptors</td>
<td>100</td>
</tr>
<tr>
<td>3.30</td>
<td>cAMP data generated at RAMP3$<em>{118-121}$ AMY$</em>{(a)}$ and WT AMY$_{(a)}$ receptors</td>
<td>101</td>
</tr>
<tr>
<td>3.31</td>
<td>cAMP data for rAmy responses at RAMP$<em>{42-45}$, RAMP$</em>{50-53}$, RAMP$<em>{58-61}$, RAMP$</em>{70-73}$, RAMP$<em>{86-89}$ and RAMP$</em>{94-97}$ AMY$<em>{(a)}$ receptors vs WT AMY$</em>{(a)}$ receptor</td>
<td>103</td>
</tr>
<tr>
<td>4.1</td>
<td>Amino acid sequence alignment of three human RAMPs</td>
<td>110</td>
</tr>
<tr>
<td>4.2</td>
<td>The RAMP1 crystal structure and RAMP3 model of the N-terminal domain showing the residues involved in generating RAMP1/3 mutants</td>
<td>110</td>
</tr>
<tr>
<td>4.3</td>
<td>ELISA data for mycRAMP1 mutants expressed with HA-CLR</td>
<td>111</td>
</tr>
<tr>
<td>4.4</td>
<td>cAMP data generated at WT CGRP and WT AM$_{2}$ receptors</td>
<td>112</td>
</tr>
<tr>
<td>4.5</td>
<td>cAMP data generated at A34E CGRP and WT CGRP receptors</td>
<td>113</td>
</tr>
<tr>
<td>4.6</td>
<td>cAMP data generated at V46D CGRP and WT CGRP receptors</td>
<td>114</td>
</tr>
<tr>
<td>4.7</td>
<td>cAMP data generated at W74E CGRP and WT CGRP receptors</td>
<td>115</td>
</tr>
<tr>
<td>4.8</td>
<td>cAMP data generated at A87P CGRP and WT CGRP receptors</td>
<td>116</td>
</tr>
<tr>
<td>4.9</td>
<td>cAMP data generated at E88L CGRP and WT CGRP receptors</td>
<td>117</td>
</tr>
<tr>
<td>4.10</td>
<td>cAMP data generated at V89A CGRP and WT CGRP receptors</td>
<td>118</td>
</tr>
<tr>
<td>4.11</td>
<td>cAMP data generated at F93I CGRP and WT CGRP receptors</td>
<td>119</td>
</tr>
<tr>
<td>4.12</td>
<td>cAMP data generated at S103N CGRP and WT CGRP receptors</td>
<td>120</td>
</tr>
<tr>
<td>4.13</td>
<td>ELISA and cAMP data generated at RAMP3$_{86-89}$ CGRP and WT CGRP receptors</td>
<td>122</td>
</tr>
</tbody>
</table>
Figure 4.14 ELISA data for mycRAMP1 mutants expressed with HA-CT .......... 123
Figure 4.15 cAMP data generated at A34E AMY1(a) and WT AMY1(a) receptors ... 124
Figure 4.16 cAMP data generated at V46D AMY1(a) and WT AMY1(a) receptors ... 125
Figure 4.17 cAMP data generated at W74E AMY1(a) and WT AMY1(a) receptors ... 126
Figure 4.18 cAMP data generated at A87P AMY1(a) and WT AMY1(a) receptors ... 127
Figure 4.19 cAMP data generated at E88L AMY1(a) and WT AMY1(a) receptors ... 128
Figure 4.20 cAMP data generated at V89A AMY1(a) and WT AMY1(a) receptors ... 129
Figure 4.21 cAMP data generated at F93I AMY1(a) and WT AMY1(a) receptors ... 130
Figure 4.22 cAMP data generated at S103N AMY1(a) and WT AMY1(a) receptors ... 131
Figure 5.1 ELISA data for the RAMP3 mutants expressed with HA-CLR .......... 136
Figure 5.2 cAMP data generated at E35A AM2 and WT AM2 receptors .......... 137
Figure 5.3 cAMP data generated at D46V AM2 and WT AM2 receptors .......... 138
Figure 5.4 cAMP data generated at E74W AM2 and WT AM2 receptors .......... 139
Figure 5.5 cAMP data generated at P87A AM2 and WT AM2 receptors .......... 140
Figure 5.6 cAMP data generated at L88E AM2 and WT AM2 receptors .......... 141
Figure 5.7 cAMP data generated at A89V AM2 and WT AM2 receptors .......... 142
Figure 5.8 cAMP data generated at I93F AM2 and WT AM2 receptors .......... 143
Figure 5.9 cAMP data generated at N103S AM2 and WT AM2 receptors .......... 144
Figure 5.10 ELISA and cAMP data generated at RAMP186-89 AM2 and WT AM2 receptors ................................................................. 146
Figure 5.11 cAMP data generated at E35A AMY3(a) and WT AMY3(a) receptors ... 148
Figure 5.12 cAMP data generated at D46V AMY3(a) and WT AMY3(a) receptors ... 149
Figure 5.13 cAMP data generated at E74W AMY3(a) and WT AMY3(a) receptors ... 150
Figure 5.14 cAMP data generated at P87A AMY3(a) and WT AMY3(a) receptors ... 151
Figure 5.15 cAMP data generated at L88E AMY3(a) and WT AMY3(a) receptors ... 152
Figure 5.16 cAMP data generated at A89V AMY3(a) and WT AMY3(a) receptors ... 153
Figure 5.17 cAMP data generated at I93F AMY3(a) and WT AMY3(a) receptors .... 154
Figure 5.18 cAMP data generated at N103S AMY3(a) and WT AMY3(a) receptors ... 155
Figure 6.1 cAMP data for hAM2 responses. ......................................................... 160
Figure 6.2 cAMP data for hAM15-52 responses. .................................................... 161
Figure 6.3 Binding data of 125I-hAM13-52 ............................................................. 163
Figure 6.4 ELISA data for RAMP3 mutants expressed with HA-CLR ............... 164
Figure 6.5 cAMP data generated at E74W AM2 and WT AM2 receptors .......... 165
Figure 6.6 cAMP data generated at E74F AM2 and WT AM2 receptors .......... 166
Figure 6.7 cAMP data generated at E74Y AM2 and WT AM2 receptors............................ 167
Figure 6.8 cAMP data generated at E74A AM2 and WT AM2 receptors ......................... 168
Figure 6.9 cAMP data generated at E74S AM2 and WT AM2 receptors ......................... 169
Figure 6.10 cAMP data generated at E74T AM2 and WT AM2 receptors ......................... 170
Figure 6.11 cAMP data generated at E74R AM2 and WT AM2 receptors ......................... 171
Figure 6.12 cAMP data generated at E74N AM2 and WT AM2 receptors ......................... 172
Figure 6.13 ELISA data for mycRAMP1 mutants expressed with HA-CLR ......................... 173
Figure 6.14 cAMP data generated at W74E CGRP and WT CGRP receptors .................... 174
Figure 6.15 cAMP data generated at W74F CGRP and WT CGRP receptors .................... 175
Figure 6.16 cAMP data generated at W74Y CGRP and WT CGRP receptors ................. 176
Figure 6.17 cAMP data generated at W74A CGRP and WT CGRP receptors ................. 177
Figure 6.18 cAMP data generated at W74N CGRP and WT CGRP receptors ................. 178
Figure 7.1 Cell-surface expression of Δ(1-47)hCT(a) and hCT(a) constructs .................... 187
Figure 7.2 Cell-surface expression of HA-CT(a) with and without mycRAMP1 .............. 188
Figure 7.3 cAMP data for rAmy responses at hAMY1(a), Δ(1-47)hAMY1(a), hCT(a) and Δ(1-
47)hCT(a) ............................................................................................................ 190
Figure 7.4 cAMP data for hCT responses at hAMY1(a), Δ(1-47)hAMY1(a), hCT(a) and Δ(1-
47)hCT(a) ............................................................................................................ 191
Figure 7.5 cAMP data for sCT responses at hAMY1(a), Δ(1-47)hAMY1(a), hCT(a) and Δ(1-
47)hCT(a) ............................................................................................................ 192
Figure 7.6 cAMP data for hαCGRP responses at hAMY1(a), Δ(1-47)hAMY1(a), hCT(a) and
Δ(1-47)hCT(a) ........................................................................................................ 193
Figure 7.7 cAMP data for hβCGRP responses at hAMY1(a), Δ(1-47)hAMY1(a), hCT(a) and
Δ(1-47)hCT(a) ........................................................................................................ 194
Figure 7.8 cAMP data for Tyrα hαCGRP responses at hAMY1(a), Δ(1-47)hAMY1(a), hCT(a)
and Δ(1-47)hCT(a) ............................................................................................... 195
Figure 7.9 cAMP data for rAmy response antagonised by sCT8,32 ............................. 197
Figure 7.10 cAMP data for rAmy response antagonised by AC187 ......................... 199
Figure 7.11 Cell-surface expression of three forms of hCT(a) ........................................ 201
Figure 7.12 cAMP data generated at the hAMY1(a) receptors formed by mycRAMP1 and
Δ(1-47)hCT(a) - I347 or Δ(1-47)hCT(a) .................................................................. 202
Figure 7.13 Summary of pEC50 values for the six peptide responses determined at hAMY1(a),
Δ(1-47)hAMY1(a), hCT(a) and Δ(1-47)hCT(a) ...................................................... 204
List of Figures

**Figure 7.14** $pK_B$ for sCT$_{8-32}$ and AC187 in antagonising rAmy at hAMY$_{1(a)}$, Δ(1-47)hAMY$_{1(a)}$, hCT$_{(a)}$ and Δ(1-47)hCT$_{(a)}$ ................................................................. 207

**Figure 8.1** The crystal structure of the RAMP1 N-terminal domain showing the functional effects induced by RAMP1/3 chimaeras ................................................................. 214

**Figure 8.2** The RAMP1 crystal structure and RAMP3 model of the N terminus showing the residues upon which mutagenesis has been performed in the literature and this thesis . 220

**Figure 8.3** The RAMP1 crystal structure and RAMP3 model of the N terminus showing residues 86-89. .................................................................................................................. 222
List of Tables

Table 1.1 Molecular composition and pharmacological profile summary for human CGRP, AM, CT and Amy receptors ........................................... 3
Table 1.2 Summary of mutagenesis data that are available in human RAMP1 from the literature ................................................................. 33
Table 1.3 Summary of mutagenesis data that are available in human RAMP2 from the literature ................................................................. 42
Table 1.4 Summary of mutagenesis data that are available in human RAMP3 from the literature ................................................................. 47
Table 3.1 Summary of cAMP data for rAmy responses at AMY_1(a) receptors containing RAMP1/3 chimaeras ........................................... 104
Table 3.2 Summary of potency changes for hβCGRP and TyrhαCGRP at RAMP1/RAMP3 chimaeras co-expressed with CT_1(a) .................... 106
Table 6.1 Summary of pEC_50 values for human RAMP3 mutants at position 74, expressed with HA-CLR ........................................... 179
Table 6.2 Summary of pEC_50 values for human RAMP1 mutants at position 74, expressed with HA-CLR ........................................... 180
Table 7.1 Summary of pEC_50 and E_max for rAmy response at hAMY_1(a), Δ(1-47)hAMY_1(a), hCT_1(a) and Δ(1-47)hCT_1(a) measured in cAMP assay ........................................... 190
Table 7.2 Summary of pEC_50 and E_max for hCT response at hAMY_1(a), Δ(1-47)hAMY_1(a), hCT_1(a) and Δ(1-47)hCT_1(a) measured in cAMP assay ........................................... 191
Table 7.3 Summary of pEC_50 and E_max for sCT response at hAMY_1(a), Δ(1-47)hAMY_1(a), hCT_1(a) and Δ(1-47)hCT_1(a) measured in cAMP assay. ........................................... 192
Table 7.4 Summary of pEC_50 and E_max for hαCGRP response at hAMY_1(a), Δ(1-47)hAMY_1(a), hCT_1(a) and Δ(1-47)hCT_1(a) measured in cAMP assay ........................................... 193
Table 7.5 Summary of pEC_50 and E_max for hβCGRP response at hAMY_1(a), Δ(1-47)hAMY_1(a), hCT_1(a) and Δ(1-47)hCT_1(a) measured in cAMP assay ........................................... 194
Table 7.6 Summary of pEC_50 and E_max for Tyr^αCGRP response at hAMY_1(a), Δ(1-47)hAMY_1(a), hCT_1(a) and Δ(1-47)hCT_1(a) measured in cAMP assay ........................................... 195
Table 7.7 pK_B values for sCT_8-32 in antagonising rAmy response at hAMY_1(a), Δ(1-47)hAMY_1(a), hCT_1(a) and Δ(1-47)hCT_1(a) measured in cAMP assay ........................................... 197
Table 7.8 pK_B values for AC187 in antagonising rAmy response at hAMY_1(a), Δ(1-47)hAMY_1(a), hCT_1(a) and Δ(1-47)hCT_1(a) measured in cAMP assay ........................................... 199
List of Abbreviations and Symbols

AM  Adrenomedullin
AM\textsubscript{1}  Adrenomedullin receptor subtype 1
AM\textsubscript{2}  Adrenomedullin receptor subtype 2
AM\textsubscript{2}  Intermedin
Amy  Amylin
AMY\textsubscript{1}  Amylin receptor subtype 1
AMY\textsubscript{2}  Amylin receptor subtype 2
AMY\textsubscript{3}  Amylin receptor subtype 3
Bqi  Becquerel
BSA  Bovine serum albumin
cAMP  Cyclic adenosine monophosphate
cGMP  Cyclic guanosine monophosphate
CGRP  Calcitonin gene-related peptide or CGRP receptor
Ci  Curie
CLR  Calcitonin receptor-like receptor
CRSP  Calcitonin receptor stimulating peptide
CT  Calcitonin
CT\textsubscript{(a)}  The insert negative variant of the human calcitonin receptor
CT\textsubscript{(b)}  The insert positive variant of the human calcitonin receptor
CTR  Calcitonin receptor
DTT  Dithiothreitol
ELISA  Enzyme-linked immunosorbent Assay
eNOS  Endothelial nitric oxide synthase
ERK  Extracellular signal-regulated kinase
ET-1  Endothelin-1
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
GIPR  Gastic inhibitory polypeptide receptor
GLP-1  Glucagon-like petidie-1
GPCR  G protein-coupled receptor
h  Human
hCT\textsubscript{(a)}  Insert-negative variant of human calcitonin receptor
IBMX  Isobutylmethylxanthine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>Inositol phosphate</td>
</tr>
<tr>
<td>IUPHAR</td>
<td>International union of basic and clinical pharmacology</td>
</tr>
<tr>
<td>J-domain</td>
<td>Juxamembrane domain</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>NHREF</td>
<td>Na(^+)/H(^+) exchanger regulatory factor-1</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide-sensitive factor</td>
</tr>
<tr>
<td>OPD</td>
<td>o-Phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>PAMP</td>
<td>Proadrenomedullin N-terminal peptide</td>
</tr>
<tr>
<td>P1R</td>
<td>Parathyroid hormone/parathyroid hormone-related peptide receptor type 1</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase-activating peptide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95/drosophila discs large/ZO-1 homology domain</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</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>preproAM</td>
<td>Preproadrenomedullin</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PTHrP</td>
<td>parathyroid hormone-related peptide</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>r</td>
<td>Rat</td>
</tr>
<tr>
<td>RAMP</td>
<td>Receptor activity modifying protein</td>
</tr>
<tr>
<td>RCP</td>
<td>Receptor component protein</td>
</tr>
<tr>
<td>rpm</td>
<td>Round per minute</td>
</tr>
<tr>
<td>s</td>
<td>Salmon</td>
</tr>
<tr>
<td>SCR</td>
<td>Short consensus repeat</td>
</tr>
<tr>
<td>SDM</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline containing 0.1% Tween20</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VPAC</td>
<td>Vasoactive intestinal polypeptide/pituitary adenylate cyclase activating peptide</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>The negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximum response</td>
</tr>
<tr>
<td>E&lt;sub&gt;max&lt;/sub&gt;</td>
<td>The maximum response that an agonist produces</td>
</tr>
<tr>
<td>pA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>The negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of the agonist needed to elicit the original response obtained in the absence of antagonist</td>
</tr>
<tr>
<td>pK&lt;sub&gt;B&lt;/sub&gt;</td>
<td>The negative logarithm to base 10 of an antagonist equilibrium dissociation constant</td>
</tr>
<tr>
<td>pIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>The negative logarithm to base 10 of the molar concentration of an unlabelled agonist that inhibits the binding of a radioligand by 50%</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

1.1 Overview

1.1.1 The calcitonin family of peptides

The calcitonin family of peptides comprises calcitonin (CT), amylin (Amy), calcitonin gene-related peptides (CGRPs), and adrenomedullin (AM) and a recently added member AM2 (also known as intermedin). Although these members show low amino acid sequence homology (Figure 1.1), their secondary structures reveal a strong correlation. The two conserved cysteines close to the N terminus form a disulphide bridge which is critical to their biological activities. Figure 1.1 shows an alignment of the primary amino acid sequences of the human (h) calcitonin family of peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>hACGRP</td>
<td>--------A-NTATVTHRLAGLLSRSGG-VKKNFVPTN-VGSKAF 37</td>
</tr>
<tr>
<td>hBCGRP</td>
<td>--------A-NTATVTHRLAGLLSRSGG-VKKNFVPTN-VGSKAF 37</td>
</tr>
<tr>
<td>hAM</td>
<td>YRQSMNFQG-RSPQ-RESGTQK-LAYQVFTD-KDKNVAPRSKIBJOQY 52</td>
</tr>
<tr>
<td>hAM2</td>
<td>TQAQLRLYV-VIQGKLNIKRLWKLMGPAGQDSAPVDPSPHCH 47</td>
</tr>
<tr>
<td>hAmy</td>
<td>--------K-NTATATQRLANFLVHSSN-NFGAILSTN-VGSNTY 37</td>
</tr>
<tr>
<td>hCT</td>
<td>--------CGLNST-MLGTYTDPFNKFHT--------FPQTAIGVGAP 32</td>
</tr>
</tbody>
</table>

Figure 1.1 Amino acid sequence alignment of the calcitonin family of peptides (human αCGRP and βCGRP, AM, AM2 (47 amino acids), Amy and CT). Amino acid sequences were obtained from GenBank, and alignment was performed using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Two cysteines that are conserved across all the peptides are highlighted in green; residues that are conserved between hAM and hAM2 are highlighted in pink; residues that are different between hαCGRP and hβCGRP are highlighted in yellow. “*” indicates the identical residues; “:” indicates the residues that possess similar side-chain property; “.” indicates the residues that have similar shapes.

1.1.2 The receptors of the calcitonin family peptides

The calcitonin family of peptides interacts with receptors belonging to the family B (secretin-like) G protein-coupled receptors (GPCR), namely calcitonin receptor (CTR) and calcitonin receptor-like receptor (CLR). These family B GPCRs were initially considered to act as monomers until the discovery of receptor activity-modifying proteins (RAMPs). In 1998, McLatchie et al. isolated a cDNA which encoded a 148-amino-acid protein during attempts
to expression-clone the gene encoding the human CGRP receptor from cells derived from a human neuroblastoma (SK-N-MC cells) (McLatchie et al., 1998). Xenopus oocytes expressing this protein gave rise to large concentration-dependent responses to CGRP, compared with the endogenous response. This protein was named RAMP1, which together with CLR reconstituted a functional CGRP receptor. The coexpression of these two components is obligatory for the full functionality of a CGRP receptor, as neither of them induced a significant response to CGRP when transfected alone into human embryonic kidney cells (HEK293T) which do not express endogenous CGRP or CT receptors (McLatchie et al., 1998). Besides human RAMP1, another two proteins belonging to the RAMP family of proteins were also cloned, which were named as RAMP2 (cloned from SK-N-MC cells) and RAMP3 (cloned from human spleen) (McLatchie et al., 1998).

The interactions between RAMPs and CLR or CTR generate different receptor phenotypes for the calcitonin family peptides. Whilst RAMP1 and CLR association constitutes a CGRP receptor, two subtypes of AM receptors, AM1 and AM2, are formed from RAMP2 and RAMP3 interactions with CLR, respectively. Amongst the calcitonin-family of peptides, CT is the only known peptide ligand that binds to its receptor with high affinity without RAMP. Co-expression of a RAMP molecule with CTR, however, generates the Amy receptors, namely AMY1, AMY2 and AMY3, based on the RAMP that they contain (Note that the abbreviation Amy denotes the peptide but AMY denotes the receptor for this peptide in this thesis). Therefore, the interaction between RAMPs and receptors confers ligand specificity. The pharmacological profiles for the cloned receptors of the calcitonin family peptides have been investigated in different cell types. Nevertheless, some receptor pharmacology remains unclear, largely due to a lack of effective and selective pharmacological tools (e.g. antagonists). Table 1.1 summarises the molecular composition and agonist pharmacological profile for the calcitonin family peptide receptors.
Table 1.1 Molecular composition and pharmacological profile summary for the human CGRP, AM, CT and Amy receptors (Hay et al., 2006b). r, rat.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Molecular</th>
<th>Pharmacological profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP receptor (CGRP)</td>
<td>CLR + RAMP1</td>
<td>hαCGRP &gt; hAM ≥ rAmy</td>
</tr>
<tr>
<td>AM receptor (AM₁)</td>
<td>CLR + RAMP2</td>
<td>hAM &gt; hαCGRP &gt; rAmy</td>
</tr>
<tr>
<td>AM receptor (AM₂)</td>
<td>CLR + RAMP3</td>
<td>hAM &gt; hαCGRP &gt; rAmy</td>
</tr>
<tr>
<td>Amy receptor (AMY₁)</td>
<td>CTR + RAMP1</td>
<td>rAmy ≥ hαCGRP &gt; hCT &gt; hAM</td>
</tr>
<tr>
<td>Amy receptor (AMY₂)</td>
<td>CTR + RAMP2</td>
<td>Poorly defined</td>
</tr>
<tr>
<td>Amy receptor (AMY₃)</td>
<td>CTR + RAMP3</td>
<td>rAmy ≥ hαCGRP &gt; hAM</td>
</tr>
<tr>
<td>CT receptor (CTR)</td>
<td>CTR alone</td>
<td>hCT &gt; rAmy, hαCGRP &gt; hAM</td>
</tr>
</tbody>
</table>

1.2 CGRP

There are two forms of CGRP: αCGRP and βCGRP. αCGRP, a 37-amino-acid peptide, is produced by differential splicing of RNA transcripts from the CT gene (Amara et al., 1982). The splicing site has been determined (Lou & Gagel, 1998). There are six exons in the pre-mRNA of the CT gene; αCGRP is synthesised when the pre-mRNA is processed to exclude exon 4 to generate a mRNA containing exons 1-3 plus 5 and 6; otherwise CT will be synthesised when the pre-mRNA is processed to include exon 4 to generate a mRNA containing exons 1-4. This RNA splicing is tissue specific. For example, in the thyroid it is CT mRNA that is found to be predominating whereas the αCGRP mRNA predominates in the hypothalamus. βCGRP is encoded by a separate gene on chromosome 11 which shows a high homology to the CT gene but does not produce CT (Steenbergh et al., 1985). Amino acid sequence analysis has shown that αCGRP and βCGRP are highly conserved; there are only three amino acids that are different between hαCGRP and hβCGRP (Figure 1.1) and one amino acid difference between the rat αCGRP and βCGRP (Steenbergh et al., 1985).

Though βCGRP is expressed in humans, mice and rats, some mammals such as pigs and cattle do not express this form of CGRP but instead express calcitonin receptor stimulating peptides (CRSP) (Katafuchi & Minamino, 2004). There are three forms of CRSPs; CRSP-1, CRSP-2 and CRSP-3 which are all produced as propeptides. These three forms of CRSPs show reasonably high sequence homology to CGRP. For example, porcine CRSP-1, -2 and -3 share 70%, 60% and 57% identity to porcine (α)CGRP, respectively. Nevertheless, CRSPs do not appear to act on the CGRP receptor (Katafuchi & Minamino, 2004).
CGRP immunoreactivity and binding have been observed throughout the central and peripheral nervous systems, which may suggest its roles in a broad range of biological processes (Poyner, 1992). The best known role of CGRP is its action in the cardiovascular system. CGRP is a potent vasodilator which relaxes the vascular smooth muscle through nitric oxide (NO)-dependent or NO-independent mechanisms (Gray & Marshall, 1992). Knockout models with either pure αCGRP deleted or both αCGRP and CT deleted showed an increase in blood pressure (Kurihara et al., 2003; Li et al., 2004a). In addition, CGRP and its receptors have been identified in the part of the brain involved in pain perception, suggesting its involvement in pain (Trang et al., 2006). It has been demonstrated that the differential behavioural responses to noxious heat observed with different mice strains (AKR vs C57BL/6) were mediated by the αCGRP levels in these mice (Mogil et al., 2005). Furthermore, CGRP also has a role in inflammation. This is supported by the observation of reduced cytokine production from dendritic cells in bone marrow in mice lacking RAMP1 (Tsujikawa et al., 2007). In addition to the processes above, CGRP has also been implicated in bone metabolism; it possibly plays a role in enhancing bone formation (Lerner, 2006; Wedemeyer et al., 2007).

The CGRP receptor has been targeted for the treatment of migraine. Increase in CGRP levels in the cranial circulation during migraine has been evidenced (Goadsby et al., 1990). Intravenous administration of CGRP triggered a delayed migrainous headache in some migraine sufferers (Lassen et al., 1998). Efforts have been therefore put into developing antagonists of the CGRP receptors. CGRP_8-37 (8-37 fragment of CGRP) is the peptide antagonist of CGRP (Chiba et al., 1989), which has been widely used for characterising the CGRP receptors. In addition, a non-peptide antagonist BIBN4096BS (olcegepant) has been developed (Doods et al., 2000). It is more potent than CGRP_8-37 and shows selectivity for human over rat receptors (Doods et al., 2000; Wu et al., 2000); it is also highly selective to the CGRP receptor over the AM or Amy receptors (Hay et al., 2006a). BIBN4096BS has been proven to be efficacious in treating acute migraine headache in human clinical trials (Olesen et al., 2004). Although BIBN4096BS shows good potency and selectivity, it requires intravenous formulation due to its high molecular weight, high polar surface area and dipeptide core (Rudolf et al., 2005). More recently, success has been made in identifying an orally active CGRP antagonist, MK-0974 (telcagepant) (Ho et al., 2008; Paone et al., 2007). MK-0974 has been shown to be a very potent antagonist of the primate CGRP receptors. It is
also highly selective for the CGRP receptors over AM receptors as demonstrated in $^{125}$I-hAM competitive binding assays (Salvatore et al., 2008).

### 1.3 AM

Human AM is a 52-amino-acid peptide, which is the longest peptide in the calcitonin family of peptides. It was first isolated from the human pheochromocytoma (Kitamura et al., 1993). Its precursor peptide was also identified which was named as preproadrenomedullin (preproAM). The preproAM gene is located on chromosome 11 (Hinson et al., 2000). In addition to AM, preproAM also gives rise to another product, namely proadrenomedullin N-terminal peptide (PAMP) and adrenotensin. However, these two peptides do not show any cross-reactivity at the AM receptors (Gumusel et al., 1996; Moody et al., 2000).

It is now known that multiple forms of AM exist. Five members of AM have been identified in teleost fish, namely AM1, AM2, AM3, AM4 and AM5 (Ogoshi et al., 2006). AM2 expression has been found in humans (Roh et al., 2004; Takei et al., 2004). There are two forms of human AM2, one is 47 amino acids long and the other has 6 extra amino acids at the N terminus.

Since the discovery of AM, its production has been identified in many cell types; for example, vascular endothelial cells, vascular smooth muscle cells, and macrophages (Kubo et al., 1998; Sugo et al., 1994a; Sugo et al., 1994b). AM is also an extremely potent vasodilator and has been suggested to play a role in homeostasis or pathophysiology of blood pressure (Nishio et al., 1997). An elevation of circulating AM levels is associated with many cardiovascular, endocrine and renal disorders (Hinson et al., 2000; Ishimitsu et al., 2006). The important role of AM in cardiovascular function has also been demonstrated in genetic models. AM$^{+/-}$ knockout mice showed an elevated blood pressure (Shindo et al., 2001) whereas blood pressure regulation was enhanced in rats with AM overexpression (Imai et al., 2001) and intravenous infusion of AM had beneficial hemodynamic effects in patients with heart failure (Nagaya et al., 2000).

Furthermore, AM is crucial for angiogenesis and lymphangiogenesis. Knockout mice lacking a functional AM gene failed to survive past midgestation (Caron & Smithies, 2001). The symptoms associated were severe generalised oedema, abnormal major vessel formation and decreased heart size. Like AM knockout mice, AM receptor deficient mice with calcr$^{+/-}$ or RAMP$^{+/-}$ knockout also died in utero at midgestation, associating with severe deformation,
edema and hemorrhage (Dackor et al., 2006; Ichikawa-Shindo et al., 2008). The importance of AM in lymphangiogenesis has also been demonstrated. The endogenous AM has been shown to be crucial for lymphatic vascular development during embryogenesis. Deletion of AM, CLR or RAMP genes was lethal; mice with these deletions died at midgestation with massive cutaneous edema due to defects in lymphatic vessel development (Fritz-Six et al., 2008). On the other hand, infusion of AM induced lymphangiogenesis and increased lymphedema in mice (Jin et al., 2008).

More recently, focus has been placed on the relevance of AM in cancer. AM has been shown to be highly expressed in a variety of human cancer cell lines such as breast, glioblastoma, pancreas and prostate lineages (Oehler et al., 2003; Ouafik et al., 2002; Ramachandran et al., 2007; Rocchi et al., 2001). AM is a mitogenic factor capable of stimulating the growth of cancer cells and has been shown to be an important tumour survival factor (Cuttitta et al., 2002). Therefore, the effect of inhibiting AM action on tumour development has been studied. It has been shown that tumour growth could be suppressed using neutralising antibodies targeting AM (in vitro, (Ouafik et al., 2002)), neutralising antibodies targeting the receptor components i.e. CLR, RAMPs 2 and 3 (in vivo, (Kaafarani et al., 2009)) or AM antagonists (in vivo, (Ishikawa et al., 2003)). These studies have suggested that AM and its receptors could be potentially targeted for the treatment of cancer.

1.4 CT

CT is the shortest peptide in the calcitonin family of peptides; it has only 32 amino acids. CT is a hormone secreted from the thyroid C cells (Sexton et al., 1999). CT peptides have been identified and isolated from many species which can be grouped into three major classes: teleost/avian, artiodactyl and rodent/human (Purdue et al., 2002). Surprisingly, salmon CT (sCT) is a more potent agonist than hCT at hCTR. It has been demonstrated that sCT and hCT bind at different sites in the hCTR in photoaffinity cross-linking studies (Dong et al., 2004b; Pham et al., 2005).

CT also has important physiological roles. CT modulates calcium homeostasis through inhibiting osteoclast mediated bone resorption (Sexton et al., 1999). Studies using knockout mice with full or partial CTR or CT gene deleted have been carried to investigate the role of CT and its receptor in regulating bone metabolism (Dacquin et al., 2004; Davey et al., 2008). As CT is known to protect against bone loss, it was interesting that knockout mice lacking the gene coding for CTR (calcr<sup>+/−</sup>) showed an increased bone formation, resulting in a high bone
mass phenotype. Further studies demonstrated that this increased bone formation was mild under normal condition, but augmented in the condition of hypercalcaemia, implicating that the regulatory effect of CT on bone formation was greater in pathological conditions. These important physiological roles make CT a very attractive therapeutic target. sCT (e.g. Miacalcin) is an approved drug for the treatment of bone disorders such as Paget’s disease, osteoporosis and hypercalcemia of malignancy.

1.5 Amy

Amy was initially discovered in amyloid deposits of human insulinoma and the pancreas of type 2 diabetic patients (Cooper et al., 1987). Human Amy is composed of 37 amino acids and has an important physiological significance. Level of circulating Amy rises after meal ingestion, and this peptide inhibits gastric emptying, gastric acid secretion, post-prandial glucagon secretion and food intake (Höppener et al., 2000). Knockout mice lacking the Amy gene showed a gain in body weight (Devine & Young, 1998; Gebre-Medhin et al., 1998). In addition, Amy is also found to oppose the metabolic actions of insulin in skeletal muscle (Sexton & Perry, 1996). A therapeutic drug has already been developed from Amy; Symlin™, a modified form of human Amy, is commercially available for the treatment of Type-1 and Type-2 diabetes. Patients taking Symlin™ in combination with standard insulin therapy injection showed lower post meal blood glucose levels and reduced glucose variability during the day compared to patients taking insulin alone. In addition, weight loss has also been reported in patients taking Symlin™.

1.6 CLR/RAMPs receptor complexes (CGRP and AM receptors)

1.6.1 Pharmacology of CGRP receptors

Since the identification of CGRP$_{8-37}$ as a CGRP antagonist (Chiba et al., 1989), it has become a widely used tool for pharmacological characterisation of the CGRP receptors. Two populations of CGRP receptors were defined based on the observations that CGRP$_{8-37}$ displayed much more potent antagonist properties toward the CGRP receptors in preparations such as guinea pig atrium or ileum compared to other preparations such as rat or guinea pig vas deferens (Dennis et al., 1990; Quirion et al., 1992). These observations led to the traditional classification of CGRP receptors into two subgroups: CGRP$_{8-37}$-sensitive CGRP$_1$ receptors (pA$_2 \geq 7$) and CGRP$_{8-37}$-insensitive CGRP$_2$ receptors (pA$_2 < 7$) (Poyner et al., 2002). In addition, the CGRP$_1$ and CGRP$_2$ receptors showed different sensitivities to the
linear CGRP analogues (Cys(ACM),\(^{2,7}\)hαCGRP and (Cys(Et),\(^{2,7}\)hαCGRP. Both of these two agonists were reported to display higher potency at the CGRP\(_2\) receptor than at the CGRP\(_1\) receptor (Dennis et al., 1989; Dumont et al., 1997).

However, the historic CGRP\(_1\):CGRP\(_2\) classification scheme has been questioned for underestimating the extent of heterogeneity for the CGRP activated receptors (Hay et al., 2004; Poyner et al., 2002). Agonists are not a good tool of choice for defining receptor subtypes, as the results are highly system-dependent and rely on the receptor density of the preparation at a given experimental time (Hay et al., 2004). This is particularly problematic with (Cys(ACM),\(^{2,7}\)hαCGRP; it has been reported to act as a partial agonist in the porcine coronary artery (Waugh et al., 1999). In addition, (Cys(Et),\(^{2,7}\)hαCGRP, which was initially considered as a CGRP\(_2\) subtype selective agonist, is in fact also an effective agonist of CGRP\(_1\) (Bailey & Hay, 2006; Nodin et al., 2005; Wu et al., 2000). Although data from experiments employing CGRP\(_{8-37}\) are more convincing than these other agonists, the situation is further complicated by the fact that wide spread of pA\(_2\) values have been observed in different preparations. This is not only seen from different species; a spread of pA\(_2\) values for CGRP\(_{8-37}\) in tissues from the same species has also been reported in many studies (Cox & Tough, 1994; Poyner, 1995). For example, pA\(_2\) for CGRP\(_{8-37}\) in different tissue preparations from the rat ranges from <5 in thoracic aorta to 8.0 in kidney, displaying over 1000-fold difference (Poyner et al., 2002).

The classification situation has only been clarified recently by International Union of Basic and Clinical Pharmacology (IUPHAR). It is now clear that the CGRP\(_2\) receptor phenotype actually results from the activation of the Amy and AM receptors by CGRP (Hay et al., 2008). CGRP has significant affinity at both AM receptors (particularly AM\(_2\)) and Amy receptors (AMY\(_1\) and AMY\(_3\)) (Hay et al., 2005; Hay et al., 2003; Hay et al., 2008; Kuwasako et al., 2004). Furthermore, both receptor types are weakly antagonised by CGRP\(_{8-37}\) (Hay et al., 2008) (Figure 1.2). Therefore, it has been clarified that there is only one type of CGRP receptor, which refers to the “CGRP\(_1\)” receptor in the old classification scheme (Hay et al., 2008). The sole CGRP receptor has molecular composition as a heterodimeric composite of CLR and RAMP1 as described in Section 1.1.2.
Figure 1.2 Apparent pA$_2$ estimates of CGRP$_{8-37}$ on human cell lines and tissues. hαCGRP was used as the agonist for these experiments (Hay et al., 2008).

1.6.2 AM receptors

AM and CGRP have overlapping biological actions, and some biological activities of AM may be mediated by the CGRP receptors. The inhibiting effect of CGRP$_{8-37}$ on both CGRP and AM induced vasodilation has been reported in different tissue locations (Hall et al., 1995; Nuki et al., 1993). However, it is clear that the AM receptor populations are located at distinct sites from the CGRP receptors in tissues. In $^{125}$I-CGRP and $^{125}$I-AM binding studies, significantly lower affinity was observed for CGRP or Amy than AM at the $^{125}$I-AM labelled binding sites (Poyner et al., 1999). Specific binding sites for AM have been reported in many cell lines such as rat-2 fibroblasts, rat skeletal muscle cell line, rat astrocytes and neuroblastoma cells (Coppock et al., 1999; Coppock et al., 1996; Zimmermann et al., 1996).

Two AM receptor subtypes have been identified based on their molecular composition: AM$_1$ (CLR/RAMP2) and AM$_2$ (CLR/RAMP3) (McLatchie et al., 1998; Muff et al., 1998; Poyner et al., 2002). Expression of RAMPs 2 and 3 does not correlate in some cells or tissues (e.g. SK-N-MC neuroblastoma, Rat-2 fibroblast and aortic smooth muscle cells, etc) (Hay et al., 2006b), indicating the preferential expression of one subtype of the AM receptor but not the other in certain tissues. In addition, receptor components of the CGRP and AM receptors can be differentially regulated in vivo in disease state (Ono et al., 2000). For example, it has been reported that CLR, RAMP1 and RAMP2 mRNA levels were upregulated in the rat with obstructive neuropathy, but RAMP3 was not affected (Nagae et al., 2000).
Chapter 1

The peptide fragment AM\textsubscript{22-52} is the only available selective antagonist which is specific for AM receptors (Eguchi \textit{et al.}, 1994). It behaves as a competitive antagonist and is able to block a number of AM activities. Hay and colleagues have shown that AM\textsubscript{22-52} has differential selectivity for AM\textsubscript{1} and AM\textsubscript{2}, and it may be possible to discriminate between these two AM receptor subtypes based on the pharmacological profiles displayed by AM\textsubscript{22-52} and CGRP\textsubscript{8-37} (Hay \textit{et al.}, 2003). Nevertheless, AM\textsubscript{22-52} is a relatively low-affinity peptide and its specificity in native systems is awaiting further characterisation. Attempts have been made to generate higher affinity antagonists (Robinson \textit{et al.}, 2009). Several smaller AM fragments, AM/αCGRP and AM/AM2 chimaeras were generated and tested for their selectivity and affinity at both AM and CGRP receptors. Of these peptides, some chimaeras displayed high affinity towards the AM receptors, however, they were non-selective over AM\textsubscript{1}/AM\textsubscript{2} and the CGRP receptor. Nevertheless, the study has revealed some key regions that could potentially become targets for further studies.

For AM2, it has been suggested that both forms of AM2 (47 and 52 amino acids) were non-selective at the CGRP or AM receptors in the transfected cells (Hay \textit{et al.}, 2005; Roh \textit{et al.}, 2004; Takei \textit{et al.}, 2004). They displayed moderate potency at these receptors. In addition, AM\textsubscript{217-47} acts as an antagonist of AM2 (Roh \textit{et al.}, 2004).

1.6.3 Intracellular signalling (CGRP and AM receptors)

The adenylate cyclase/cAMP (cyclic adenosine monophosphate) system has been identified as the major signal transduction pathway for CGRP and AM. Elevation of cAMP levels upon either CGRP or AM stimulation has been reported in both native tissues and cell lines transfected with cloned receptors (Hinson \textit{et al.}, 2000; Muff \textit{et al.}, 1995; Poyner, 1997). The pathways in most cases compose a common cascade via GPCR activation of G\textsubscript{\alpha}s, adenylate cyclase activation, subsequent increase in cAMP and activation of protein kinase A (PKA). This CGRP/AM induced PKA activation has various biological effects including the best known action of these peptides as vasodilators (Brain & Grant, 2004; Champion \textit{et al.}, 2003). Besides G\textsubscript{\alpha}s coupling, There is also evidence for CGRP and/or AM to couple to G\textsubscript{\alpha}o (Disa \textit{et al.}, 2000; Main \textit{et al.}, 1998) (Armstead & Vavilala, 2007; Sakai \textit{et al.}, 1998) and G\textsubscript{\alpha}q/11 (Drissi \textit{et al.}, 1998; Morara \textit{et al.}, 2008) to conduct signal. However, the coupling to these G\textsubscript{\alpha} subunits seems to be dependent on the cellular background and some biological effects of these peptides may be contributed by signal transductions mediated by different G protein subunits.
AM shows effects on cell growth and mitogenesis. This has led to investigations on mitogen-activated protein (MAP) kinases. Results obtained from these studies seem to be dependent on both cell type and MAP kinase type. For example, studies in mesangial cells showed that AM inhibited endothelin-1 (ET-1)-stimulated MAP kinase but stimulated expression of a MAP phosphatase (Haneda et al., 1996; Hinson et al., 2000). The MAP kinase pathway is also involved in CGRP signalling. It has been demonstrated to be important for CGRP-mediated cell proliferation (Yu et al., 2009) and neural function (Wang et al., 2009).

Protein kinase B/Akt may also be involved in CGRP and AM signalling (Nikitenko et al. 2006). AM-induced angiogenesis was reported to occur through the activation of phosphatidylinositol 3-kinase (PI3K)/Akt, extracellular signal-regulated kinase (ERK) and tyrosine phosphorylation of focal adhesion kinase (p125FAK) (Kim et al., 2003). In addition, it has been suggested that AM induced endothelium-dependent vasodilation at least partially via the NO/cyclic guanosine monophosphate (cGMP)-mediated pathway (Hayakawa et al., 1999; Nishimatsu et al., 2001; Shindo et al., 2000). It has been demonstrated that the production of cGMP was dependent on the PI3K/Akt-mediated pathway in the rat aorta (Nishimatsu et al., 2001). AM might activate endothelial NO synthase (eNOS) by stimulating the direct binding of Ca$^{2+}$/calmodulin to eNOS, which in turn induces a conformational change to eNOS and subsequent activation of eNOS. This activation of eNOS is thought to be mediated through the phosphorylation by Akt, a downstream target of PI3K. Furthermore, it has been shown that AM infusion significantly attenuated myocardial ischemia/reperfusion injury in the rat and the effects were mainly resulted from antiapoptotic effects of AM via the PI3K/Akt-dependent pathway (Okumura et al., 2004).

1.7 CTR and CTR/RAMP receptor complexes (CT and Amy receptors)

1.7.1 CTR

The first CTR clone was isolated by Lin and colleagues from a cDNA library derived from the porcine renal epithelia cell line (LLC-PK1) in 1991 (Lin et al., 1991). Its expression has been subsequently identified in many different tissues across multiple species. Amino acid sequences reveal reasonably high homology across species. Human CTRs (hCTR) share ~78% and 67% amino acid sequence identity with rodent CTRs and porcine CTRs, respectively (Purdue et al., 2002). Amongst the family B GPCRs, CTR has the highest sequence homology to CLR (~55% in humans. However, unlike CLR which requires RAMPs for cell-surface expression, the expression of CTR is independent of RAMP; it has been shown to be
effectively transported to the cell surface on its own in all cell lines that have been studied including CHO-P (Chinese hamster ovary-P cells), Cos 7 (African green monkey kidney cells), RAEC (rat aortic endothelial cells), HEK293 cells (Morfis et al., 2008; Muff et al., 1999; Tilakaratne et al., 2000; Zumpe et al., 2000).

1.7.1.1 Receptor heterogeneity

The existence of multiple CTR subtypes was initially suggested from the early radioligand binding study in rodent brain membrane preparations, where high and low affinity binding sites of a non-helical sCT analogue were observed (Nakamuta et al., 1990). Since then, numerous CTR isoforms have been cloned from different species. For instance, a study using reverse transcriptase-PCR analysis showed that there were at least seven splice variants arising from alternate splicing within the 5’ region of the CTR gene in the mouse (Anusaksathien et al., 2001). The underlying mechanism was not fully defined, but it might be related to the alternate utilisation of separate promoters (Anusaksathien et al., 2001).

In humans, at least six hCTR splice variants have been described to date (Poyner et al., 2002). Amongst them, hCT\textsubscript{a} (also known as hCTR\textsubscript{1} or hCTR2) and hCT\textsubscript{b} (also known as hCTR\textsubscript{1+} or hCTR1) are the two most common isoforms of hCTR. These amino acid sequences are identical except that hCT\textsubscript{b} has an additional 16 amino-acids insert in the first intracellular loop. Although hCT\textsubscript{a} is more widely expressed, there is little difference observed between these two variants in terms of CT recognition ability (Moore et al., 1995; Nussenzveig et al., 1994). Nevertheless, these two variants do display significantly different signalling properties (See Section 1.7.3). Two additional hCTR variants arise from alternative splicing within the 5’ untranslated regions. The other two hCTR variants exist in the coding sequence, with one variant leading to a stop codon within the first intracellular loop and the other lacking 47 amino acids at the N-terminus (Albrandt et al., 1995; Gorn et al., 1992; Moore et al., 1995). Characterisations performed on the latter variant suggested that the N-terminal truncation had little impact on the receptor function. It displayed high affinity \textsuperscript{125}I sCT binding and responded to hCT with increases in cAMP level (Albrandt et al., 1995). However, these observations were from the very early study; better characterisation is required to define the pharmacology of these hCTR variants. Such characterisation could potentially bring more insights to our understanding of ligand to receptor binding mechanisms.
Distinct splice variants of rodent and rabbit CTRs have also been reported, however, no orthologues appear to be expressed in human (Kuestner et al., 1994).

The identification of polymorphic CTR variants further complicates the receptor classification. In 1997, a polymorphism was located at amino acid 463 of hCT\(_{(b)}\) (447 in hCT\(_{(a)}\)) by Nakamura and colleagues using restriction fragment length polymorphism analysis (Nakamura et al., 1997). This polymorphism generates either a leucine or a proline at the site. The prevalence of the homozygotes and heterozygotes of this polymorphism appears to vary in different ethnic groups (Masi et al., 1998; Nakamura et al., 1997; Taboulet et al., 1998).

1.7.1.2 Receptor pharmacology

CTRs display high affinity towards CT, in contrast to lower affinity towards CGRP, Amy and AM (Sexton et al., 1999). CTR-mediated peptide responses can be effectively antagonised by the 8-32 fragment of sCT, sCT\(_{8-32}\), but not CGRP\(_{8-37}\) or AM\(_{22-52}\) (Poyner et al., 2002).

Peptides exhibit differential potency and affinity at CTRs of different species. For example, baby hamster kidney derived cell lines expressing cloned hCT\(_{(a)}\) display equal accumulation of cAMP upon stimulation of sCT or hCT, but hCT is 3- to 10-fold lower in affinity than sCT at hCT\(_{(a)}\) in competing for \(^{125}\)I-sCT binding (Houssami et al., 1995). In contrast, hCT is weaker in both stimulation of cAMP production (~10-fold) and competing for \(^{125}\)I-sCT binding (100- to 1000-fold) than sCT in HEK293 cell lines expressing rCTR (Houssami et al., 1995). These variations in peptide binding and response might reflect differences in structures of CTRs in different species, which may in turn change the micro-environment around the binding domains (Purdue et al., 2002).

Porcine CTR on the other hand exhibits different binding properties. Amy displayed high affinity and potency at porcine CTR in the absence of RAMP cotransfection (Houssami et al., 1994; Sexton et al., 1994). This is in contrast to the observation of Amy being a very weak agonist at rCTR or hCTR in the absence of RAMPs. However, this work is in line with the study conducted by Kikumoto and colleagues, where porcine CTR in conjunction with RAMPs did not give a higher affinity for Amy than porcine CTR alone (Kikumoto et al., 2003). The reasons for these observations are still unclear, but it has been suggested that the default conformation of porcine CTR might be somehow equivalent to CTRs from other species dimerised with RAMPs (Purdue et al., 2002).
1.7.2 Amy receptors

Identification of the Amy receptors in vivo has been proven to be difficult due to the lack of specific antibodies. There is yet no direct evidence on the molecular identity of the Amy receptors in vivo, however, it is commonly accepted that complexes formed by CTR and RAMPs are most likely to resemble the receptor compositions in native tissues. This hypothesis is supported by the observations that reconstitutions of the Amy receptors by coexpression of CTR and RAMPs show very similar pharmacological profiles to those found in native tissues (Poyner et al., 2002).

1.7.2.1 Receptor heterogeneity

The current IUPHAR classification scheme for the Amy receptors is AMY$_1$, AMY$_2$ and AMY$_3$, corresponding to the Amy receptors formed by CTR with RAMPs 1, 2 and 3, respectively. These subtypes can be further classified according to the CTR splice variant present, such as AMY$_{1(a)}$ (CT$_{(a)}$/RAMP1) and AMY$_{1(b)}$ (CT$_{(b)}$/RAMP1) (Poyner et al., 2002). However, the pharmacological properties of these Amy receptors can sometimes vary considerably between different host cellular environments. This variance might result from the presence of endogenous CLR and RAMP components in some tissues as well as some unknown cellular factors such as G protein contents (see Section 1.7.3) (Christopoulos et al., 1999; Tilakaratne et al., 2000). To date, there is still no reliable means which can effectively distinguish between the Amy receptor subtypes or the Amy receptors from CTR. Nevertheless, Hay and colleagues have shown that it is possible to discriminate these receptors by careful use of combinations of agonists and antagonists, and have identified such spectra of agonists and antagonists (see below) (Hay et al., 2005).

1.7.2.2 Receptor pharmacology

Differential receptor phenotypes are observed in different cell lines. Whilst coexpression of hCT$_{(a)}$ with RAMP1 or RAMP3, but not RAMP2, generates an Amy receptor phenotype in Cos 7 cells, high affinity Amy binding was observed at hCT$_{(a)}$ with all three RAMPs in CHO-P. In contrast, hCT$_{(b)}$ was found to induce high affinity Amy binding with all three RAMPs in both cell lines (Tilakaratne et al., 2000). Actions of other members of the calcitonin family of peptides at the Amy receptors also vary amongst different cell lines. For example, AM was shown able to stimulate the Amy receptors formed by hCT$_{(a)}$ with all three RAMPs in HEK293 cells, but such stimulation was not observed with any of the three RAMPs in
complexes with hCT\textsubscript{(a)} in Cos 7 cells (Christopoulos et al., 1999; Hay et al., 2005; Kuwasako et al., 2003b; Udawela et al., 2006a).

Subtypes of the Amy receptors show peptide differential specificity. In general, both AMY\textsubscript{1} and AMY\textsubscript{3} have higher affinities for sCT and Amy but lower affinities for hCT (Hay et al., 2005; Poyner et al., 2002). Furthermore, various affinities for CGRP have been reported with the Amy receptors. Of note, AMY\textsubscript{1(a)} exhibits a CGRP\textsubscript{2}-like pharmacology, according to the old classification scheme, (Hay et al., 2005; Kuwasako et al., 2004). This receptor has a relatively high affinity for CGRP and can be weakly antagonised by CGRP\textsubscript{8-37} (Hay et al., 2005).

Peptide responses at the Amy receptors can be antagonised using sCT\textsubscript{8-32} (Leuthauser et al., 2000). However, in terms of discriminating between the Amy receptor subtypes, sCT\textsubscript{8-32} is not a good choice, as it shows high affinity for all three Amy receptor subtypes with little discrimination between them (Hay et al., 2005). In addition, sCT\textsubscript{8-32} does not discriminate strongly between the CT and Amy receptors. On the other hand, two other selective antagonists named AC187 (Young et al., 1994) and AC413 (Wookey et al., 1996) are more effective. AC187 and AC413 are peptide chimaeras of rAmy and sCT\textsubscript{8-32}. They display selectivity between CTR and Amy receptor subtypes (Hay et al., 2005). AC187 is more potent (~10-fold) at AMY\textsubscript{3(a)} over CT\textsubscript{(a)} in Cos 7 cells, whereas AC413 is moderately selective (~7-fold) for AMY\textsubscript{1(a)} over AMY\textsubscript{3(a)} (Hay et al., 2005). Nevertheless, neither of them alone is particularly useful for discrimination between these receptors; Amy receptor subtypes can only be discriminated by carefully selecting multiple antagonists in combination with some agonists (Hay et al., 2005).

### 1.7.3 Intracellular signalling (CTR and Amy receptors)

CTR is linked to multiple signal transduction pathways. The coupling of CTR to \(G_\alpha_s\) leading to the activation of adenylate cyclase and PKA pathway has been identified in various systems. Elevation of cAMP levels upon stimulation of CT occurs in both Cos cells transfected with cloned CTR as well as in mouse \(\alpha\)-TSH cells (thyrotropic cells) which have an endogenous expression of CTR (Moore et al., 1995; Perry et al., 1997).

CTR also activates the phospholipase C/protein kinase C (PLC/PKC) pathway via coupling to \(G_\alpha_q\), leading to the production of inositol phosphate (IP) and mobilisation of intracellular \(Ca^{2+}\) (Force et al., 1992; Kuestner et al., 1994; Moore et al., 1995). Investigations with the
two major hCTR splice variants suggest differential signal transduction pathways for them. Compared to hCT\(_{(a)}\), hCT\(_{(b)}\) does not only show a loss of G\(\alpha_q\)-mediated response, its ability of coupling to G\(\alpha_s\) is also significantly reduced (Moore \textit{et al}., 1995). It is possible that the 16 amino acid insert in the first intracellular loop in hCT\(_{(b)}\) has distorted receptor interactions with G\(\alpha\) proteins.

Activation of other signalling pathways by CTR has also been reported. Chen and colleagues found a transient activation of mitogen-activated protein (MAP) kinase pathway via coupling of CTR to G\(\alpha_i\) in HEK293 cells (Chen \textit{et al}., 1998). Such coupling has also been seen with the rodent receptors (Pozvek \textit{et al}., 1997). In addition, other CTR-mediated responses including activation of phospholipase D and some tyrosine kinases have been reported (Purdue \textit{et al}., 2002).

The selectivity of G protein coupling and subsequent signal transduction by CTR is not only splice variant-specific, but also determined by other cellular elements, at least to some extent. For instance, Chakraborty and colleagues showed that CTR was selectively coupled to different G\(\alpha\) proteins at different stages of cell cycle, with stronger activation of G\(\alpha_s\) in G2 phase and activation of G\(\alpha_q/G\alpha_q\) at S phase (Chakraborty \textit{et al}., 1994).

Unlike the relatively well characterised CTR, signal transduction events for the Amy receptors are poorly investigated. The Amy receptor-mediated responses are also primarily linked to G\(\alpha_s\) and G\(\alpha_q\) coupling. A study investigating the relative coupling of the Amy receptors to cAMP generation, intracellular Ca\(^{2+}\) and ERK1/2 activation in Cos 7 cells showed that the Amy receptors formed by CT\(_{(a)}\) and RAMPs are coupled to G\(\alpha_q\) to a less extent, compared to CT\(_{(a)}\) transfected alone into cells (Morfis \textit{et al}., 2008). Further examination has also been performed in HEK293. Consistent with Cos 7 cells, there was a stronger induction of Amy receptors/G\(\alpha_q\)-mediated cAMP signalling compared to the induction of ERK and Ca\(^{2+}\) signalling. However, the observation of greater induction of Ca\(^{2+}\) signalling compared to the ERK signalling in HEK293 cells was the reversed pattern seen in Cos 7 cells. These inconsistent observations in HEK293 and Cos 7 cells suggest the possibility of some cell dependent modulation of the Amy receptor mediated signalling (Morfis \textit{et al}., 2008). Furthermore, the presence of RAMPs also contributes to the Amy receptor signalling. A study using RAMP1/2 chimaeras identified a crucial role of the C-terminal domain of RAMPs in the signalling by the Amy receptors containing CT\(_{(a)}\) in Cos 7 cells; the signalling is potentially via G protein or regulatory protein interaction (Udawela \textit{et al}., 2008).
al., 2006a). It has been demonstrated that the C-terminal domain of RAMPs is crucial for Amy receptor coupling to G proteins (Udawela et al., 2006a). Deletion of the extreme C terminus of each RAMP led to attenuated Amy receptor phenotype induction with loss of Amy binding in Cos 7 cells. However, the loss could be partially restored with overexpression of Gαs protein, suggesting that the RAMP C terminus might be involved in G protein coupling to the Amy receptors (see section 1.9.4).

Overall, the selectivity of transduction pathways by CTR and Amy receptors is dependent on cell type, splicing variant and some unknown cellular elements. Whether the selective G protein coupling by CTR and Amy receptors is due to a higher coupling efficiency of these receptors to certain G protein subunit than to the alternate subunits, or due to differential cellular densities of G protein subunits is still unclear (Purdue et al., 2002).

1.8 Ligand binding to the calcitonin peptide family receptors

1.8.1 GPCRs

GPCRs can be subdivided into three main families: family A (rhodopsin/β2 adrenergic receptor-like receptors), family B (e.g. calcitonin peptide family receptors) and family C (e.g. GABAB, which are characterised by an exceptionally long amino terminus ranged from 500 to 600 amino acids) (Gether, 2000). Alternatively, GPCRs can also be grouped using GRAFS classification system which is based on phylogenetic studies (Schiöth & Fredriksson, 2005). There are five main families, namely Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, and Secretin (GRAFS). The calcitonin peptide family receptors belong to the secretin family in this classification scheme. Of note, the former classification scheme is preferred in this thesis.

Family A GPCRs are so far best characterised: the organisation of the seven transmembrane (TM) helices has been deduced from a low-resolution structure of frog rhodopsin by cryo-electron microscopy (Unger et al., 1997), followed by the successful resolution of its crystal structure (Palczewski et al., 2000). The understanding of family A GPCRs have been further augmented by the publications of the ligand-bound avian and human β1/β2 adrenoceptors and the human A2A adenosine receptor (Cherezov et al., 2007; Jaakola et al., 2008; Rasmussen et al., 2007; Rosenbaum et al., 2007; Warne et al., 2008). In contrast, less is known about the family B GPCRs; to date, no structure has been determined for any full-length receptor from this family. Human family B GPCRs comprise 15 members including a variety of peptide hormones and neuropeptides such as calcitonin, vasoactive intestinal peptide (VIP),
parathyroid hormone (PTH) and glucagon (Hoare, 2005). Family B GPCRs are characterised by a long extracellular N-terminal domain (approximately 100-160 amino acids) and a juxtamembrane domain (J-domain) of seven TM α-helices which is a common structural feature for all GPCRs (Figure 1.3) (Hoare, 2005).

**Figure 1.3** A schematic diagram showing a common structure shared by family B GPCRs. The N-terminal, J-domain and C-terminal ends are labelled in the figure. The most prominent characteristic of the family B GPCRs is a large extracellular amino terminus containing several cysteines, forming a network of disulphide bridges (Gether, 2000).

### 1.8.2 Mechanisms of peptide interaction with family B GPCRs

Evidence for the ligand-receptor interactions of family B GPCRs comes mainly from the data generated from structure-activity relationship analyses by mutagenesis, photoaffinity cross-linking studies, and more recently, from researchers who have been able to predict more detailed structures of receptor fragments using nuclear magnetic resonance (NMR). Sites involved in the ligand-receptor interaction can be accommodated into the structure using NMR chemical-shift perturbation. A general mechanism of peptide binding, termed “two-domain model” has been proposed for family B GPCRs; step 1, the C terminus of the peptide ligand binds to the N terminus of receptor to induce an affinity trap where the local peptide concentration elevates enormously in the vicinity of the J-domain; step 2, the N-terminus of ligand binds to the J-domain of the receptor which leads to the activation of G protein (Hoare, 2005).

The existence of the two functional domains in the family B GPCRs was first illustrated in studies employing chimaeras of peptides and receptors. Bergwitz and colleagues showed that a chimaera containing the N-terminal portion of salmon CT and C-terminal portion of bovine
PTH selectively stimulated the chimaeric receptor made of the C-terminal portion of porcine CTR and N-terminal portion of rat PTH receptor, but not the wild type (WT) receptors or the reciprocal chimaeric receptor (to a very low level) (Bergwitz et al., 1996). This selective activation was also observed with the reciprocal ligand and receptor chimaeras. This N to C domain binding orientation between the receptor and ligand has also been seen with the chimaeric receptors of glucagon/glucagon-like peptide-1 (GLP-1), CT/glucagon, secretin/VIP and VIP/pituitary adenylate cyclase-activating peptide (PACAP) (Holtmann et al., 1995; Laburthe et al., 2002; Stroop et al., 1995).

Studies employing photoaffinity cross-linking further support the two domain models and provide some insight into the residues involved in the ligand-receptor interaction. It has been reported that a photolabile residue within the carboxyl-terminal half (residue 26) and mid-region (residue 16) of hCT covalently labelled the extracellular amino-terminal domain of its receptor (Dong et al., 2004b). Similar studies have also been carried out for the VIP receptor; photoreactively labelled residues at positions 22 and 24 of VIP cross-linked to residues G116 and C122 of the N terminus of VPAC-1, respectively (Tan et al., 2006).

In the two domain model, the J-domain is responsible for the activation of the G proteins. This role has been demonstrated experimentally. A mutated receptor containing only the J-domain of the PTH receptor could still be stimulated to a similar maximum response as the full length receptor, but much lower potency was observed as a result of lacking the of N-terminal interaction (Shimizu et al., 2001). Furthermore, mutations introduced to the J-domains can lead to a constitutive activation of the receptors (Gaudin et al., 1998; Hjorth et al., 1998). The ligand binding to the family B GPCRs is also likely to induce a conformational change within the J-domain of the receptor. This has been evidenced in a photoaffinity cross-linking study, where a photoreactively labelled residue of an inverse agonist could cross-link to two different sites in the J-domain of the human PTH/parathyroid hormone-related peptide (PTHrP) receptor type 1 (P1R) (Gensure et al., 2001).

### 1.8.3 Structure of family B GPCRs and their ligands

A NMR study by Grace et al. resolved the structure of the major ligand binding domain of the mouse corticotrophin-releasing factor (CRF) receptor 2β, a soluble protein comprising amino acids 39-133 of the extracellular N-terminal domain (ECD1-CRF-R2β) (Grace et al., 2004). The structure has been identified as a short consensus repeat (SCR) containing two antiparallel β sheets. It is stabilised by three disulphide bonds and interconnected by a central...
core consisting of a salt bridge sandwiched by two aromatic rings. The two β sheets are flanked by two disordered regions (residues 39-58 and 84-98) and the central core is further surrounded by a layer of highly conserved residues (Grace et al., 2004). The NMR structure of ECD₁-CRF-R2β complexed with a peptide antagonist (astressin) was later published (Grace et al., 2007b). The structure showed that the hydrophobic face of the C-terminal helix of a stressin interacted with the SCR motif of the receptor, further supporting the two domain model proposed for family B GPCRs.

Another NMR study has resolved the solution structure of a complex formed by PACAP and the N-terminal domain of its receptor (human splice variant PAC1-Rs) (Sun et al., 2007). The structure suggested that the N-terminal structure of PAC1-Rs adopted a similar organisation as CRF-R2β, but with an additional N-terminal helix and a short extended strand after the fourth β sheet.

The structure of the J-domain and C terminus for family B GPCRs remains unclear. A few studies have made homology models of the TM domains of PTH receptors based on the rhodopsin crystal structure (Gensure et al., 2003; Monticelli et al., 2002). A more recent study has built a model of gastric inhibitory polypeptide receptor (GIPR) at the atomic level using a comparative approach using those known structures of other GPCRs (Malde et al., 2007). The overall arrangement of the TM domain of GIPR predicted is similar to that of bovine rhodopsin; the TM domain formed by a circular bundle of seven helices and interconnected with extracellular or intracellular loops of no regular secondary structures. These regions are stabilised by the conserved disulphide bond between the first and second extracellular loops (Malde et al., 2007). The C terminus of GIPR is predicted to consist of two α-helices (residues 403-406 and 423-430) connecting to a globular head at the end of the C-terminus (Malde et al., 2007).

Unlike the receptors, the structures of the peptide ligands for the family B GPCRs have been relatively better characterised. The peptides mainly have α-helices as backbones. This has been shown both in the aqueous solutions (Mierke & Pellegrini, 1999; Ying et al., 2003) and in the crystal structures of PTH and glucagon (Jin et al., 2000; Sasaki et al., 1975). In addition, a study published by Grace et al. determined the NMR structures of six CRF-related peptides (Grace et al., 2007a). They generally share a common “helix-loop-helix” structure with a small kink or a turnaround residue in region 25-27.
1.8.4 Residues of CLR and CTR important for ligand to receptor interactions

Studies on the family B GPCRs mentioned above suggest the crucial role of the N-terminal domain of the receptors in the interactions with ligands. In common with other family B GPCRs, the N termini of CLR and CTR are apparently important for the interactions with the calcitonin family peptides. A recent NMR analysis has been performed on a soluble form of the extracellular domain of the CGRP receptor comprising the N-terminal residues 23-133 of CLR and residues 26-117 of RAMP1 (Koth et al., 2010). This complex was still able to bind to CGRP (though with a lower affinity), confirming the importance of the N-terminal domains for ligand binding.

Nevertheless, there is only limited data available on the regions of CLR involved in the interactions with either ligands or RAMPs. An early study using chimaeras made between mouse CLR and PTH1 receptor demonstrated that region 23-60 at the extreme N-terminal domain of CLR was important for its association with RAMP1 (Ittner et al., 2005). A recent study has performed an alanine scan in this region to investigate the role of the individual residues (Barwell et al., 2010). hαCGRP potency and binding and receptor internalisation were determined for these alanine mutants. The study suggested that Q45 and Y49 may be involved in the RAMP1 interactions whereas L41, A44, I32, G35, T37 may be involved in the CGRP interactions. In addition, L24 and L34 at the extreme N-terminus have been demonstrated to interact with the C-terminal amidated F37 of CGRP; alanine mutations at these two residues affected CGRP binding but not receptor activation (Banerjee et al., 2006).

Deletion mutations have been made at the N-terminal domain of CLR. Deletion of the N-terminal 18 residues of human CLR abolished CGRP and AM binding and potency at the CGRP and AM₁ receptors, respectively. Fusion of the N-terminal 24 amino acids of the porcine CTR to the deleted region was able to restore the CGRP response or partially restore the AM response (Koller et al., 2002). In another study, deletion of residues 36-42 of mouse CLR reduced AM binding at the AM₁ receptor but CGRP binding was not affected at the CGRP receptor (Koller et al., 2004).

Extracellular cysteine residues of CLR (C212 and C225 in the first and C282 in the second extracellular loops) have been mutated to alanine (Kuwasaki et al., 2003b). The C212A and C282A mutations induced a reduction in AM potency; it was suggested these two cysteines might form a disulphide bond.
Furthermore, some CLR residues have been demonstrated to be important for non-peptide CGRP antagonists. Amino acids 37-63 in the N terminus of CLR have been identified to be responsible for high affinity binding of a class of non-peptide antagonists closely related to BIBN4096BS using CLR/CTR chimaeras (Salvatore et al., 2006). More recently, a mutagenic screen (mostly mutated to alanine) was performed in the N-terminal region 23-63 of CLR (Miller et al., 2010). The study identified residue M42 to be important for high affinity interactions of antagonists BIBN4096BS and MK-0974; M42A significantly reduced the affinities of both antagonists at the CGRP receptor (Miller et al., 2010).

Little is known about the residues of CTR that are involved in the ligand binding. Photoaffinity labelling experiments have shown that photolabile residues incorporated at positions 8, 16, and 26 of hCT covalently labelled residues L368, F137 and T30 of hCTR, respectively (Dong et al., 2004a, 2004b). A similar study identified that region 134-141 of human CTR was covalently linked to position 19 of sCT (Pham et al., 2004). Consistent with sCT, position 19 of antagonist sCT\textsubscript{8-32} was later shown to also cross-link to region 134-131 (Pham et al., 2005). In addition, the cross-linking site of position 8 of sCT\textsubscript{8-32} was demonstrated to locate at M49 of hCTR (Pham et al., 2005). This is different from the site identified for hCT where position 8 was cross-linked to L368 on hCTR (Dong et al., 2004b), suggesting the possible difference in the binding pockets for hCT and sCT.

Progressive truncations performed from the N-terminal residue 47 of hCTR demonstrated the importance of the N terminus to the hCT response (Dong et al., 2009). hCT potency was reduced for these truncated mutants and the response was completely abolished after truncation of 114 residues. In addition, the study has demonstrated that small molecule ligands bind to distinct regions of CTR to hCT; region 150-153 was suggested to be critical for these small molecules (Dong et al., 2009).

Whilst CLR and CTR are crucial for ligand binding, the RAMP is also an important component for the calcitonin family of peptides interactions with the receptors. That distinct receptor phenotypes are resulted from the RAMP to CLR/CTR associations strongly suggests the importance of RAMPs to the receptor pharmacology. The role of RAMP in the interactions with ligands and receptors has been demonstrated in numerous studies and these will be discussed in the following section.
1.9 RAMP family of proteins

1.9.1 Other receptors interacting with RAMPs

The modulatory role of RAMPs is not limited to the calcitonin family receptors. At least four other receptors have been identified that also interact with RAMPs. They are the VIP/PACAP (VPAC-1) receptor with all three RAMPs, the glucagon and PTH-1 receptors with RAMP2 and the PTH-2 receptor with RAMP3 (Christopoulos et al., 2003). Further investigations on the VPAC-1/RAMP complexes suggested that whilst the association of RAMP had no impact on the receptor pharmacology, PI hydrolysis was enhanced at the VPAC-1/RAMP2 complex compared to VAPC-1 alone. There are also some experimental data suggesting that co-expression of RAMPs may alter β-adrenoceptor pharmacology (Hay et al., 2006b).

Furthermore, it is possible that RAMP1 and RAMP3 might be associated with a family C GPCR, the calcium sensing receptor. This association seems to be required for receptor trafficking (Bouschet et al., 2005). Indeed, being ubiquitously expressed in many cells, it will not be surprising to identify RAMPs coupled with additional proteins. For example, an interaction between RAMP1 and β-tubulin has been reported, suggesting broader biological roles for RAMPs than as GPCR accessory proteins (Kunz et al., 2007). The fact that RAMPs are more widely distributed across cell and tissue types than CTR or CLR strongly supports additional roles for RAMPs in other cellular processes (McLatchie et al., 1998).

1.9.2 Tissue distribution

RAMPs are widely distributed in human tissues. This has been demonstrated using northern blotting on mRNA derived from different human tissues (McLatchie et al., 1998). RAMP1 was expressed in uterus, bladder, brain, pancreas and gastro-intestinal tract. RAMPs 2 and 3 had similar tissue distributions and are expressed highly in lung, breast, immune system and fetal tissues. Recently, the presence of RAMP3 in human visceral adipose tissue has been experimentally demonstrated (Bailey et al., 2010). The function of RAMP3 in this tissue is still waiting to be determined.

The distribution of RAMPs has been extensively studied in rodent brain (Li et al., 2004b; Oliver et al., 2001; Ueda et al., 2001). RAMP1 was detected with a strong signal by in situ hybridisation in olfactory tubercules, nucleus accumbens, caudate putamen, cortex, amygdala and spinal cord in rat brain. RAMP1 was also present in some other parts of brain but at lower levels including claustrum, hippocampus, thalamus, hypothalamus and superior
colliculus. RAMP2 was found in many regions of rat brain, including the hippocampus, hypothalamus, amygdala, olfactory tubercules, cortex, substantia nigra compacta and the ventral horn of spinal cord. In contrast, the expression of RAMP2 in mouse brain was lower. However, notable RAMP2 expression has been detected in the choroid plexus of the third, fourth and lateral ventricles and blood vessels of the pia mater in the mouse. In contrast to the wide distribution of RAMP3 in humans, RAMP3 has relatively limited distribution in the rat; it was mainly found in lung, kidney, spleen and spinal cord (Chakravarty et al., 2000; Nagae et al., 2000). The distribution of RAMPs and the pharmacology in different cells and tissues have been extensively reviewed by Hay et al. (Hay et al., 2006b).

1.9.3 Structure of RAMPs

The primary amino acid sequences of three human RAMPs reveal ~31% identity and ~56% similarity (Figure 1.4). All three human RAMPs are proposed to share a common structure: a short intracellular C terminus (~9 amino acids), a single TM domain (21 amino acids) and a long extracellular N terminus (~118 amino acids for RAMP1 and RAMP3, ~145 amino acids for RAMP2). In addition, there is a predicted signal peptide of ~21-41 amino acids at the N terminus of RAMPs. Across species, the TM regions show high conservation, whereas the N termini are less conserved. This higher degree of variability in the N terminus suggests that this region is more likely to contribute to the pharmacological specificity that these proteins engender. Despite low N-terminal sequence homology, all RAMPs across species contain four conserved cysteine residues, which presumably form disulphide bonds, suggesting a common secondary structure.
Figure 1.4 Amino acid sequence alignment of three human RAMPs. The predicted signal peptide is in shown in blue and the TM domain is shown in red. Four conserved cysteine residues are highlighted in green. "*" indicates the identical residues; ":" indicates the residues that possess similar side-chain properties; ":" indicates the residues that have similar shapes.

Before any RAMP structure was available, attempts were made to predict the structural characteristics of RAMPs. The first human RAMP1 model was made using an ab initio modelling protocol which was developed for the structural prediction of RAMP family proteins (Simms et al., 2006). The secondary structure was obtained by a consensus from two prediction routines. They were selected based on their abilities to predict the secondary structures of 16, diverse, small, helical, disulphide-bond containing peptides of known structures. The structure predicted from this ab initio model suggested that the human RAMP1 N terminus comprised three α-helices, helix 1, helix 2 and helix 3 and that residues 118-139 form a small TM domain. The disulphide bonding pattern predicted by this model was experimentally tested using site-directed mutagenesis (SDM) at cysteine residues where the arrangement of disulphide bonds was determined using double mutants of every cysteine pair (Simms et al., 2006).

Another RAMP structural model was built later by Benítez-Páez from a primary sequence alignment of 38 RAMPs (1-3) from different species that were retrieved from a PSI blast search in NCBI with the human RAMP1 sequence lacking the signal peptide (Benítez-Páez, 2006). The model predicted in this study had a similar organisation to the ab initio model generated by Simms and colleagues (Simms et al., 2006), but with some differences including the disulphide bonding pattern and the orientation of helix 3. The author later published a follow-up study where phylogenetic and statistical analyses were carried out on the RAMPs across different species (Benítez-Páez & Cárdenas-Brito, 2008). The study
suggested a collection of residues that could potentially be functionally important, many of which were found in the extracellular domains of RAMPs. The TM domain of RAMP3 was also modelled in this study which was shown to adopt a helical structure. Residues that might be important for receptor function were suggested from this TM helix model (Benítez-Páez & Cárdenas-Brito, 2008).

The tri-helical organisation stabilised by three disulphide bonds of the human RAMP1 N terminus was confirmed by the crystal structure (residues 27-107 (RAMP127-107)) (Kusano et al., 2008). As illustrated in Figure 1.5a, the structure indicates that helix 1 is formed by residues E29 to V51 and is kinked at residue L39. It is followed by a small helical structure consisting of E53 to L55 which sits within the loop connecting helices 1 and 2. Helix 2 is anti-parallel to the other two helices, starting at W59 and ending at L80, whereas helix 3 is formed by residues from A87 to Y100. The interacting residues forming the three disulphide bonds predicted by Simms et al. (Simms et al., 2006) were confirmed in the crystal structure as C27-C82, C40-C72 and C57-C104. The structure is maintained by multiple hydrophobic interaction sites, which mostly locate between helices 1 and 2.

![Figure 1.5](image_url)

**Figure 1.5** (a) The crystal structure of the extracellular N-terminal domain of human RAMP1. The putative CLR interacting residues identified on the crystal structure are shown in red whereas the putative CGRP interacting residues are shown in yellow (Kusano et al., 2008). (b) The human RAMP3 N-terminal domain modelled by Bailey et al. (Bailey et al., 2010).
Based on the electrostatic potential distribution on the solvent-accessible-surface of the crystal structure of the human RAMP1 N terminus, a hydrophobic patch located on helices 2 and 3 was identified (Kusano et al., 2008). Residues that are likely to constitute the receptor (CLR) and ligand (CGRP) binding interfaces were proposed from this area. F93, H97 and F101 were proposed to form the CLR binding interface, whereas residues R67, D71, W74, E78 and W84 were predicted to constitute part of the ligand (CGRP) binding pocket (Figure 1.5a). The residues identified to form the CLR binding interface in the crystal structure are highly conserved in RAMP1 across different species, supporting the notion that these residues have an important role in RAMP1 function. The contribution of some of these residues to either receptor or ligand interaction has been tested experimentally; these studies are discussed below. In particular, W74 and W84 have been demonstrated to play an important role in small molecule antagonist binding to the CGRP receptor (Hay et al., 2006a; Mallee et al., 2002; Moore et al., 2010; Salvatore et al., 2006). Nevertheless, further experimental evidence is needed to confirm these assignations made based on the crystal structure.

Despite the low sequence identities between three RAMPs, they are believed to adopt a similar fold. This prediction is supported by the sequence comparison which shows that the two disulphide bonds which have a more important role in stabilising the RAMP1 structure (C40-C72 and C57-C104) are conserved across three RAMPs (Figure 1.4). Furthermore, the equivalent sites in RAMPs 2 and 3 to the intramolecular-interaction sites identified in the crystal structure of RAMP1 N-terminal domain are also mostly hydrophobic (Kusano et al., 2008). A homology model for human RAMP328-107 has been produced based on the RAMP1 crystal structure (Bailey et al., 2010; Qi et al., 2010) (Figure 1.5b). This model has provided an indication of how the structures of RAMP1 and RAMP3 compare but a crystal structure of RAMP3 is needed to confirm similarities and differences between the actual structures of these proteins. The predicted RAMP3 N-terminal structure revealed a more unwound helix 1 compared to RAMP1 and the C-terminal portion of helix 3 orientates differently due to the presence of a kink in the RAMP3 model. Although it is difficult to infer the exact shape of the loops in RAMP in protein complex due to their high flexibility, the loop between helix 2 and helix 3 of the RAMP3 model appears to be significantly different from the region in the RAMP1 structure. The apparent structural differences between the RAMP1 and RAMP3 N termini suggest that there could be differences between the parts of the binding pockets contributed by RAMPs 1 and 3.
1.9.4 Residues of RAMPs involved in ligand to receptor interactions

It has been suggested that the N terminus of RAMPs contributes to the peptide binding together with the N terminus of CLR. Cross-linking study using $^{125}$I-AM and $^{125}$I-CGRP showed that the RAMPs located close to the peptide binding pocket in the CLR/RAMP complexes (Hilairet et al., 2001b). The importance of the N-terminal domains of RAMPs in determining receptor pharmacology has been demonstrated in many studies (Fraser et al., 1999; Udawela et al., 2006b; Zumpe et al., 2000). Whilst the N terminus of RAMPs plays a crucial role in receptor pharmacology, the TM domain and C terminus are also important. The TM and the short C terminus of RAMPs do not appear to contribute directly to receptor pharmacology but instead have different functions. Chimaeras generated by swapping the TM and/or C terminus between RAMPs 1 and 2 suggested that the TM and C terminus have little effect on receptor function, but is important for RAMP to CTR interactions (Udawela et al., 2006b; Zumpe et al., 2000). In addition, progressive truncations from the C terminal end of RAMP1 including the partial or complete removal of the TM region showed that the TM domain was required for the formation of a functional CGRP receptor at the cell surface (Fitzsimmons et al., 2003; Steiner et al., 2002).

The C terminus of RAMP possesses some functional features. Human RAMP1 has an endoplasmic reticulum retention signal at its C terminus which comprises residues QSKRT (Steiner et al., 2002). The sequence is adjacent to the TM domain of RAMP1 and acts as a signal for intracellular retention. The association of CLR or CTR presumably overrides the sequence and therefore RAMP1 is transported to the cell surface when complexes with these receptors. In human RAMP3 the last four residues (DTLL) at the C terminus provide a type-1 PSD-95/Discs-large/ZO-1 homology domain (PDZ) recognition sequence. Deletion and point mutations demonstrated that the PDZ motif interacted with N-ethylmaleimide-sensitive factor (NSF) and subsequently altered receptor trafficking to recycling (Bomberger et al., 2005a). Whilst the RAMP3/CLR receptor complex can be efficiently recycled following internalisation, RAMPs 1 and 2 which both lack a PDZ recognition site cannot interact with NSF and the receptor complexes containing them are thus targeted for degradation (Bomberger et al., 2005a; Hilairet et al., 2001a; Kuwasako et al., 2000). In addition, it has been shown that the PDZ motif in RAMP3 also interacts with a protein called Na$^+/H^+$ exchanger regulatory factor-1 (NHERF-1) and this interaction appeared to be critical for the RAMP3/CLR complex internalisation (Bomberger et al., 2005b).
Furthermore, the intracellular C terminus of RAMPs is also directly involved in the Amy receptor-mediated signalling. Mutagenesis data from RAMP1/2 chimaeras with the C termini exchanged have suggested that the C terminus is responsible for Amy-evoked cAMP accumulation potency, whereas the N terminus is responsible for Amy binding affinity (Udawela et al., 2006a). The action of RAMP in receptor signalling might be through G protein coupling. C-terminal deletion mutations of RAMPs caused a reduction in Amy binding which could be partially reversed by overexpressing G-protein (Gαs) in Cos 7 cells (Udawela et al., 2006a). Further investigations showed that the three RAMPs had differential G protein coupling preference in the Amy receptors (Morfis et al., 2008). Overexpression of different Gα subunits had different impacts on the high affinity Amy binding to the receptor complexes formed by each RAMP and CT(a). In contrast, RAMPs do not seem to play a similar role in the RAMP/CLR complexes. C-terminal deletions of individual RAMPs had little impact on the CGRP or AM receptor-mediated signalling profile in Cos 7 or HEK293 cells (Udawela et al., 2006a). It has been speculated that the presence of another accessory protein of the CGRP and AM receptor, namely receptor component protein (RCP), may have taken over the RAMPs’ involvement in the CGRP and AM receptor-mediated signalling (Prado et al., 2001; Sexton et al., 2009). RCP is an intracellular peripheral membrane protein that is found associated with the RAMP/CLR complexes; it has been demonstrated to be important for Gαs-mediated cAMP signalling for these receptor complexes (Prado et al., 2001). However, the signalling role for RCP has not been reported with the Amy receptors.

Mutagenesis data available for RAMPs in the literature are summarised in Tables 1.2, 1.3 and 1.4; data generated at the N-terminal domains are discussed in the sections below.

1.9.4.1 RAMP1

Amongst the three human RAMPs, RAMP1 is the best understood member, mainly with CLR, as in the CGRP receptor. The importance of the RAMP1 N terminus in conferring receptor pharmacology was first demonstrated in a study employing RAMP1 chimaeras (Fraser et al., 1999). The respective roles of individual residues or regions within the human RAMP1 are inferred from subsequent studies employing individual amino acid substitutions, deletion mutants and chimaeras between RAMP1 and RAMP2 or RAMP3. Overall, the mutations that induced a change in either CGRP receptor function or expression are mostly located in helices 2 and 3 of the RAMP1 N terminus, which suggests that helices 2 and 3 are the major determinants of CLR and CGRP interactions (e.g. Y66, L69, T73, H97; see below).
Chapter 1

The mutagenesis data generated in the RAMP1 N terminus from the literature are summarised in Table 1.2.

1.9.4.1.1 Helix 1 and Loop 1 (joining helices 1 and 2) with CLR; CGRP receptor

Attempts have been made to identify important regions within helix 1 and loop 1 using deletion or chimaera mutants. Several such mutants (deletions 28-33, 34-39, 41-45, 46-50, 51-55 and RAMP1/2 chimaera 27-50) generally resulted in reduced peptide (hαAM and hαCGRP) potencies, which were mostly paralleled by some degree of loss in receptor expression at the cell surface (Kuwasako et al., 2001; Kuwasako et al., 2003a). SDM has also been employed to study the role of individual residues in these regions of RAMP1. These mutants and their effects are summarised in Table 1.2. Apart from the mutations introduced at two cysteine residues (C40 and C57) which showed a reduced CGRP potency and reduction in receptor cell-surface expression, the rest of the point mutations introduced in these regions elicited little effect in either CGRP response or receptor cell-surface expression. The data together indicate that helix 1 of RAMP1 may have only a minor role in directly contributing to CLR or CGRP interactions.

1.9.4.1.2 Helix 2 and Loop 1 (joining helices 1 and 2) with CLR; CGRP receptor

Several small deletion mutants covering regions 59-65, 67-71, 74-76, 78-80 and 83-86 in helix 2 and loop 2 have been characterised for their effects on CGRP and AM responses (Kuwasako et al., 2001; Kuwasako et al., 2003a). Though the data generated from these mutants are difficult to interpret as the change in pharmacology may have partially been a result of loss in cell surface expression, it has been suggested that residues 78-80 may specifically contribute to AM pharmacology as the CGRP receptor containing the deletion had a greater loss in AM than CGRP responses (Kuwasako et al., 2003a). Region 78-80 resides at the end of helix 2 and residues within this region lie either on or close to the ligand binding pocket proposed from the crystal structure of the RAMP1 N terminus. Thus, the differential effects observed with this deletion mutant on AM and CGRP responses may suggest different contributions of these residues to the AM and CGRP binding pockets, either in a direct or indirect manner.

The CGRP-interacting residues proposed from the crystal structure of the RAMP1 N terminus have been mutated to alanine (Moore et al., 2010). Amongst them, only W84A induced significant change to CGRP potency which was accompanied with a reduced 30
receptor expression at the cell surface. In addition, W84 appeared to have a broader role as it has been implicated in both peptide and non-peptide antagonist binding to the CGRP receptor in the study (Moore et al., 2010). Besides W84, another two residues identified on the crystal structure, W74 (Hay et al., 2006a; Mallee et al., 2002; Moore et al., 2010; Salvatore et al., 2006) and R67 (Moore et al., 2010) have also been demonstrated to be important for high affinity binding of small molecule antagonists at the CGRP receptor. A recent NMR analysis of the unliganded and olcegepant-bound states of the extracellular domain complex of CLR/RAMP1 has further confirmed this role of W84 and W74 (Koth et al., 2010).

Some neighbouring residues of the proposed CGRP binding interface showed the importance of this region to CGRP receptor function. Mutant Y66A almost completely abolished CLR trafficking, suggesting that considerable structural perturbation was introduced by the mutation and that Y66 may also directly contribute to the CLR binding interface (Simms et al., 2009). In contrast, L69A and T73A reduced CGRP potency without affecting the cell surface expression, indicating that they may contribute to CGRP interactions, most likely in an indirect manner given their position (not solvent exposed) in the crystal structure.

1.9.4.1.3 Helix 3 and region joining helix 3 and the TM domain with CLR; CGRP receptor

A study employing deletion mutants generated within helix 3 and region joining helix 3 and TM domain (88-90, 91-94, 96-100, 101-103, 105-107, 109-112 and 113-118) has suggested that 91-103 might be important for conferring high affinity CGRP binding whereas residues 88-90 may specifically contribute to AM pharmacology (Kuwasako et al., 2003a). Interestingly, when locating these residues in the crystal structure, residues 91-103 overlie the proposed CLR interaction site. This indicates that the change in pharmacology observed was more likely to be related to the loss in the cell surface expression (as reported in the original study) resulting from disruption of the CLR binding interface. Region 88-90 resides at the beginning of helix 3, opposite but still in close proximity to the proposed ligand binding pocket by the crystal structure. Thus the region may contribute to the AM binding pocket in either a direct or indirect manner.

Numerous point mutations which cover most of the residues in helix 3 and region joining helix 3 and the TM domain have been characterised (Table 1.2). These data provide experimental support for some of the residues that have been suggested to be important for CLR or CGRP interactions from the RAMP1 crystal structure. F93A RAMP1 in one study
induced a significant reduction in cell-surface expression of the CGRP receptor, but the CGRP response was not changed at this mutant receptor (Simms et al., 2009). In another study, reduced total CGRP binding was further observed at F93A RAMP1/CLR (Kuwasako et al., 2003a). It therefore seems unlikely that F93 is directly involved in constituting a CLR binding interface as suggested by (Kusano et al., 2008). H97, which was also predicted to locate at the CLR binding interface by the crystal structure, caused a significant reduction in both cell-surface expression and CGRP-evoked cAMP production when mutated to alanine (Simms et al., 2009). In another study, H97A led to a significantly reduced total CGRP binding, and small reductions in CGRP potency and cell-surface expression (Kuwasako et al., 2003a). These observations are broadly consistent with each other, generally supporting the importance of H97 in CLR association. Another residue at the proposed CLR binding interface, F101, showed an interesting response when mutated to alanine. Cell-surface expression and CGRP binding were significantly reduced, accompanied by a reduced maximum response of CGRP-evoked cAMP production, however, CGRP potency was not affected (Kuwasako et al., 2003a; Simms et al., 2009). It is likely that F101A reduced the ability of RAMP1 to associate with CLR which supports the involvement of F101 in constituting part of the CLR binding interface, however, the receptor function seemed to be retained or even enhanced at F101A mutant receptors.

1.9.4.1.4 RAMP1/CTR; AMY1 receptor

In contrast to CLR, the RAMP1/CTR complex is relatively less well characterised. Only a limited number of SDM data are available (Table 1.2) and very few residues have been identified to be important for ligand-receptor interaction. The proposed residues to form part of the ligand binding pocket including R67, D71, E78, W74 and W84 have been recently mutated individually to alanine (Gingell et al., 2010). Amongst these mutants, only W84A induced a significant reduction in rAmy and hαCGRP potency at AMY1(a), which was paralleled by a reduced receptor expression at the cell surface. Further substitution W84F was able to restore some of the receptor function but not receptor expression, suggesting that W84 may be important for peptide to receptor interactions in AMY1(a). The study has also suggested that the loop between helices 2 and 3 plays a key role in ligand to receptor interactions. Furthermore, W74 does not appear to be involved in the ligand to receptor interactions in AMY1(a), W74A and W74K mutations did not affect rAmy potency (Gingell et al., 2010; Hay et al., 2006a).
Table 1.2 Summary of mutagenesis data that are available in human RAMP1 from the literature. Mutants generated in various studies are grouped based on the positions of the mutations in the RAMP1 crystal structure. *Significance is not known; data presented in paper is either not sufficient, i.e. no proper controls were included or statistical analysis was not performed with the right controls. Therefore, the changes indicated do not all achieve statistical significance. CRR: corresponding region; R1: hRAMP1; R2: hRAMP2; CSE: cell-surface expression. SDM: site directed mutagenesis; N: N terminus; TM: transmembrane domain; C: C terminus; DM: double mutation; $E_{\text{max}}$: maximum response (not significantly changed unless specified in the table); ↔: unchanged compared to WT; ↓: reduced compared to WT; ↑: increased compared to WT; ↓↓: reduced to basal level; ≈: equivalent to. [1]: Fraser et al., 1999, [2]: Kuwasako et al., 2001, [3]: Kuwasako et al., 2003a, [4]: Simms et al., 2006, [5]: Simms et al., 2009, [6]: Gingell et al., 2010, [7]: Moore et al., 2010, [8]: Hay et al., 2006a, [9]: Zumpe et al., 2000, [10]: Udawela et al., 2006a, [11]: Fitzsimmons et al., 2003, [12]: Steiner et al., 2002, [13]: Kuwasako et al., 2006.

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**Loop 1**

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**Helix 2**

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**Loop 2**

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| 83-86     | Deletion     | CLR | ↓hαCGRP (10nM), ↓hAM (10nM), ↓CSE | HEK293 | [3] |
| W84       | SDM: W84A     | CT&lt;sub&gt;(a)&lt;/sub&gt; | ↓hαCGRP, ↓rAmy, ↓CSE | Cos 7 | [6] |</p>
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**Helix 3**

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The TM domain and C-terminal domain

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**The TM domain and C-terminal domain**

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<tr>
<th>TM + C</th>
<th>Chimaera: CRR of R2</th>
<th>CT\text{a}</th>
<th>rAmy binding (\approx) R2, rAmy, hαCGRP, hCT and sCT affinity (\approx) R1</th>
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</thead>
<tbody>
<tr>
<td><strong>TM + C</strong></td>
<td>Chimaera: CRR of R2</td>
<td>CT\text{a}</td>
<td>↓rAmy binding</td>
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<tr>
<td>TM</td>
<td>Chimaera: CRR of R2</td>
<td>CT\text{a}</td>
<td>↓rAmy binding, hαCGRP, rAmy, hCT and sCT potency (\approx) R1</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>Chimaera: CRR of R2</td>
<td>CT\text{a}</td>
<td>↓rAmy binding, hαCGRP, rAmy, hCT and sCT affinity (\approx) R1, hαCGRP, rAmy, and sCT potency (\approx) R2, ↓hCT potency</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>Deletion</td>
<td>CLR</td>
<td>CGRP response (\approx) R1</td>
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HEK293 and A201
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<th>TM + C</th>
<th>Chimaera: TM + C(1-9) of the PDGF receptor</th>
<th>CLR</th>
<th>↓CGRP potency and affinity</th>
<th>HEK293ts and A201 [11]</th>
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<tr>
<td>TM + C</td>
<td>Deletion</td>
<td>CLR</td>
<td>↓CGRP potency</td>
<td>HEK293ts and A201 [11]</td>
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<tr>
<td>C(1-4)</td>
<td>Deletion</td>
<td>CLR</td>
<td>Cos 7 cells: ↑hαCGRP potency, ↓CSE TSA cells: ↔hαCGRP binding</td>
<td>Cos 7 and TSA [12]</td>
</tr>
<tr>
<td>C(1-8)</td>
<td>Deletion</td>
<td>CLR</td>
<td>Cos 7 cells: ↔ hαCGRP potency, ↑CSE (in absence of CLR) TSA cells: ↔hαCGRP binding</td>
<td>Cos 7 and TSA [12]</td>
</tr>
<tr>
<td>C</td>
<td>Deletion</td>
<td>CLR</td>
<td>Cos 7 cells: ↓hαCGRP potency, ↓E&lt;sub&gt;max&lt;/sub&gt;, ↑CSE (in absence of CLR) TSA cells: *↓hαCGRP binding</td>
<td>Cos 7 and TSA [12]</td>
</tr>
<tr>
<td>C(1-10)</td>
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<td>CLR</td>
<td>Cos 7 cells: ↓hαCGRP potency, ↑CSE (in absence of CLR) TSA cells: ↓hαCGRP binding</td>
<td>Cos 7 and TSA [12]</td>
</tr>
<tr>
<td>C(1-10) + artificial RXR ER retention/ retrieval motif</td>
<td>Deletion</td>
<td>CLR</td>
<td>Cos 7 cells: ↔hαCGRP potency TSA cells: ↔hαCGRP binding</td>
<td>Cos 7 and TSA [12]</td>
</tr>
<tr>
<td>C(1-16)</td>
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<td>CLR</td>
<td>Cos 7 cells: ↓hαCGRP potency, ↑CSE (in absence of CLR) TSA cells: ↓↓hαCGRP potency, ↓receptor complex formation, ↓CLR glycosylation</td>
<td>Cos 7 and TSA [12]</td>
</tr>
<tr>
<td>C(1-29)</td>
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<td>CLR</td>
<td>Cos 7 cells: ↓hαCGRP potency, ↓↓CSE TSA cells: ↓↓hαCGRP binding, ↓↓receptor complex formation, ↔CLR glycosylation</td>
<td>Cos 7 and TSA [12]</td>
</tr>
<tr>
<td>C(1-31) + artificial RXR ER retention/ retrieval motif</td>
<td>Deletion</td>
<td>CLR</td>
<td>↓hαCGRP responses</td>
<td>Cos 7 [12]</td>
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<tr>
<td></td>
<td>Deletion</td>
<td>GFP-CLR</td>
<td>CSE</td>
<td>HEK293</td>
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<tr>
<td>-----</td>
<td>----------</td>
<td>---------</td>
<td>-----</td>
<td>--------</td>
</tr>
<tr>
<td>C</td>
<td>Deletion</td>
<td>GFP-CLR</td>
<td>↓Tyr(^\text{h(\alpha)CGRP affinity, ↔CSE, ↔h(\alpha)CGRP potency, ↓Tyr(^\text{h(\alpha)CGRP potency}}))</td>
<td>HEK293</td>
</tr>
<tr>
<td>C</td>
<td>Chimaera: CRR of R2</td>
<td>GFP-CLR</td>
<td>↔CSE</td>
<td>HEK293</td>
</tr>
<tr>
<td>C(1-9)</td>
<td>Deletion</td>
<td>CLR</td>
<td>Cos 7 cells: ↔hAM and h(\alpha)CGRP potency, ↓hAM potency, HEK293 cells: ↔hAM and h(\alpha)CGRP potency</td>
<td>Cos 7 and HEK293</td>
</tr>
<tr>
<td>C(1-9)</td>
<td>Deletion</td>
<td>CT(_{(a)})</td>
<td>↓rAmy and h(\alpha)CGRP binding, ↓h(\alpha)CGRP and rAmy potency, ↔hCT and sCT potency</td>
<td>Cos 7</td>
</tr>
</tbody>
</table>
1.9.4.2 RAMP2

1.9.4.2.1 RAMP2/CLR; AM₁ receptor

The structure-function relationships in the human RAMP2 N terminus are less well defined. The mutagenesis data generated in the RAMP2 N terminus from the literature are summarised in Table 1.3. Unlike RAMP1, the interpretation of these mutagenesis data generated at RAMP2 residues is constrained by the lack of structural model for RAMP2 at the present time.

Consistent with RAMP1, large chimaeras involving exchanging the entire N terminus of between RAMPs 1 and 2 suggest that the receptor pharmacology is mainly determined by the N terminus of RAMP2 (Fraser et al., 1999). Data generated from some smaller RAMP1/2 chimaeras have narrowed down the pharmacology-determining region to 77-101 (Kuwasako et al., 2001). Some deletion mutants within this region were also studied (deletions 66-69, 70-75, 76-78, 79-82, 83-85, 86-89, 86-92, 90-92, 93-96, 97-99 and 100-103); however, changes observed with the functional data were mostly paralleled by poor cell surface expression of the mutant AM₁ receptors (Kuwasako et al., 2001). This suggests that these mutations may have introduced gross alterations in the overall receptor structure. Nevertheless the authors speculated that region 86-92 was critical for peptide binding (Kuwasako et al., 2001). An alanine scan was additionally performed at residues 86-92, but none of the individual mutations caused a change in the AM-evoked response (Table 1.3). In addition, the equivalent region in rat RAMP2 (93-99) has been demonstrated to be important for AM affinity and potency in the rat AM₁ receptor (Kuwasako et al., 2002). Deletion of this region had a significant impact on both AM binding and potency when co-expressed with CLR.

SDM has also been carried out to investigate the role of individual residues in RAMP2 to AM₁ receptor function. The importance of the extracellular cysteine residues has been demonstrated by alanine substitution. The four mutant receptors containing C68A, C84A, C99A and C131A RAMP2 all failed to be transported and expressed at the cell surface and thus failed to respond to AM (Kuwasako et al., 2003b). This is likely to be due to destabilisation of the RAMP2 structure. Besides cysteines, the extracellular histidine residues have also been studied for their involvement in AM binding (Kuwasako et al., 2008). H124A and H127A seemed to alter the overall protein structure which led to poor cell surface expression, and failure to bind or respond to AM. H124 which is conserved among the RAMP family (H97 in RAMPs 1 and 3) is suggested to be directly involved in forming the
interaction site with CLR, similar to H97 (Kusano et al., 2008). H127 is also likely to contribute to the CLR binding interface, as it resides in close proximity to H124; the mutation at this position may have altered and thus disrupted the interface. On the other hand, H102A had little effect on the receptor expression and function. H71A displayed reduced AM binding and potency in the absence of a change in the cell surface expression. It is therefore possible that H71 in RAMP2 may be involved in AM binding, either directly or indirectly.

The equivalent residue to W74 of RAMP1, E101 in RAMP3 has been mutated to Trp (Qi et al., 2008). When coexpressed with CLR, the mutation appeared to be detrimental to both receptor expression and function. The mutant receptor was not detected at the cell surface at a level that was significantly higher than CLR alone and generated essentially no response in cAMP experiments to hAM, hαCGRP or hβCGRP. The data may suggest that E101 of RAMP2 plays an important role in the interactions with CLR. Alternatively, it is also possible that the Trp substitution introduced at position 101 had severely affected the overall protein folding which led to the subsequent failure in CLR association and receptor trafficking.

1.9.4.2.2 RAMP2/CTR; AMY$_2$ receptor

The only mutagenesis data available with CTR is for mutant E101W RAMP2 (Qi et al., 2008). E101W AMY$_2$(a) showed equivalent rAmy, hαCGRP and hβCGRP responses to the WT receptor. However, rAmy potency at WT AMY$_2$(a) was only slightly higher than at CT$_1$(a) alone in Cos 7 cells in the study. Thus the lack of effect in peptide responses seen at with the mutant receptor may have been a false negative as the Cos 7 cell system has been previously reported to generate attenuated AMY$_2$(a) phenotypes (Christopoulos et al., 1999).
**Table 1.3** Summary of mutagenesis data that are available in human RAMP2 from the literature. *Significance is not known; data presented in paper is either not sufficient, i.e. no proper controls were included or statistical analysis was not performed with the right controls. Therefore, the changes indicated do not all achieve statistical significance. CRR: corresponding region; R1: hRAMP1; R2: hRAMP2; CSE: cell-surface expression. SDM: site directed mutagenesis; N: N terminus; TM: transmembrane domain; C: C terminus; $E_{\text{max}}$: maximum response (not significantly changed unless specified in the table); ↔: unchanged compared to WT; ↓: reduced compared to WT; ↑: increased compared to WT; ↓↓: reduced to basal level; ≈: equivalent to. [1]: Fraser et al., 1999, [2]: Kuwasako et al., 2001, [3]: Kuwasako et al., 2003b, [4]: Kuwasako et al., 2008, [5]: Qi et al., 2008, [6]: Zumpe et al., 2000, [7]: Udawela et al., 2006a, [8]: Kuwasako et al., 2006, [9]: Bomberger et al., 2005.

<table>
<thead>
<tr>
<th>Position</th>
<th>Mutation</th>
<th>Receptor</th>
<th>Effect</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Chimaera: CRR of R1</td>
<td>CLR</td>
<td>AM and αCGRP response ≈ R1</td>
<td>Xenopus oocytes</td>
<td>[1]</td>
</tr>
<tr>
<td>N</td>
<td>Chimaera: CRR of R1</td>
<td>CLR</td>
<td>AM and αCGRP response ≈ R1</td>
<td>HEK293T</td>
<td>[1]</td>
</tr>
<tr>
<td>36-76</td>
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<td>CLR</td>
<td>hAM and hαCGRP potencies ≈ R2</td>
<td>HEK293</td>
<td>[2]</td>
</tr>
<tr>
<td>36-100</td>
<td>Chimaera: CRR of R1</td>
<td>CLR</td>
<td>↓↓hAM potency, ↓↓hαCGRP potency, ↓↓CSE</td>
<td>HEK293</td>
<td>[2]</td>
</tr>
<tr>
<td>36-124</td>
<td>Chimaera: CRR of R1</td>
<td>CLR</td>
<td>hAM and hαCGRP potency ≈ R1</td>
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<td>[2]</td>
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<tr>
<td>C68</td>
<td>SDM: C68A</td>
<td>CLR</td>
<td>↓↓specific hAM binding, ↓↓hAM potency, ↓↓CSE</td>
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<td>[3]</td>
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<td>CLR</td>
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<td>[3]</td>
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<td>SDM: C99A</td>
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<td>[3]</td>
</tr>
<tr>
<td>C131</td>
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<td>CLR</td>
<td>↓↓specific hAM binding, ↓↓hAM potency, ↓↓CSE</td>
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<td>[3]</td>
</tr>
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<td>66-69</td>
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</tr>
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<td>CLR</td>
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<td>↔→hαCGRP (100nM), ↔→hAM (100nM), ↔→specific hAM binding, ↔→CSE</td>
<td>HEK293</td>
<td>[2]</td>
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<td>[2]</td>
</tr>
<tr>
<td>83-85</td>
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<td>HEK293</td>
<td>[2]</td>
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<td>HEK293</td>
<td>[2]</td>
</tr>
<tr>
<td>86-92</td>
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<td>CLR</td>
<td>↓↓hAM potency, ↓↓specific hAM binding, ↓↓hAM affinity, ↓CSE</td>
<td>HEK293</td>
<td>[2]</td>
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<tr>
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<td>[2]</td>
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<tr>
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<td>↓↓hαCGRP (100nM), ↓↓hAM (100nM), ↓↓specific hAM binding, ↓↓CSE</td>
<td>HEK293</td>
<td>[2]</td>
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<td>Chimaera: CRR in R1</td>
<td>CLR</td>
<td>↓↓hAM potency, ↓↓hαCGRP potency</td>
<td>HEK293</td>
<td>[2]</td>
</tr>
<tr>
<td>100-103</td>
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<td>CLR</td>
<td>↓↓hαCGRP (100nM), ↓↓hAM (100nM), ↓↓specific hAM binding, ↓↓CSE</td>
<td>HEK293</td>
<td>[2]</td>
</tr>
<tr>
<td>H71</td>
<td>SDM: H71A</td>
<td>CLR</td>
<td>↓specific hAM binding, ↓hAM potency, ↔CSE</td>
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<td>[4]</td>
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<tr>
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<td>SDM: W86A</td>
<td>CLR</td>
<td>↔hAM potency</td>
<td>HEK293</td>
<td>[2]</td>
</tr>
<tr>
<td>M88</td>
<td>SDM: M88A</td>
<td>CLR</td>
<td>↔hAM potency</td>
<td>HEK293</td>
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<tr>
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<td>SDM: I89A</td>
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<td>↔hAM potency</td>
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<td>SDM: S90A</td>
<td>CLR</td>
<td>↔hAM potency</td>
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<td>SDM: R91A</td>
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<td>↔hAM potency</td>
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<tr>
<td>E101</td>
<td>SDM: E101W</td>
<td>CLR</td>
<td>↓hαCGRP response, ↓hβCGRP response, ↓↓hAM response, ↓↓CSE</td>
<td>Cos 7</td>
<td>[5]</td>
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<tr>
<td>H102</td>
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<td>*↔specific hAM binding, ↔hAM potency, ↔CSE</td>
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</tr>
<tr>
<td>H124</td>
<td>SDM: H124A</td>
<td>CLR</td>
<td>↓↓specific hAM binding, ↓↓hAM potency, ↓↓CSE</td>
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<tr>
<td>H127</td>
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<td>CLR</td>
<td>↓↓specific AM binding, ↓↓hAM potency, ↓↓CSE</td>
<td>HEK293</td>
<td>[4]</td>
</tr>
</tbody>
</table>

**The TM and C-terminal domain**

<p>| TM + C | Chimaera: CRR of R1 | CT&lt;sub&gt;(a)&lt;/sub&gt; | rAmy binding ≈ R1, rAmy, hCT and sCT affinities ≈ R2, ↑hαCGRP affinity | Cos 7 | [6] |</p>
<table>
<thead>
<tr>
<th>TM + C</th>
<th>Chimaera: CRR of R1</th>
<th>CT&lt;sub&gt;(a)&lt;/sub&gt;</th>
<th>↑rAmy binding ≈ R1</th>
<th>Cos 7</th>
<th>[7]</th>
</tr>
</thead>
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<tr>
<td>TM</td>
<td>Chimaera: CRR of R1</td>
<td>CT&lt;sub&gt;(a)&lt;/sub&gt;</td>
<td>↑rAmy binding, rAmy and sCT affinity ≈ R2, hαCGRP, rAmy, hCT and sCT potency ≈ R2</td>
<td>Cos 7</td>
<td>[7]</td>
</tr>
<tr>
<td>C</td>
<td>Chimaera: CRR of R1</td>
<td>CT&lt;sub&gt;(a)&lt;/sub&gt;</td>
<td>rAmy binding ≈ R2, hαCGRP, rAmy, hCT and sCT potency ≈ R1</td>
<td>Cos 7</td>
<td>[7]</td>
</tr>
<tr>
<td>C</td>
<td>Deletion</td>
<td>CLR</td>
<td>↓AM binding and affinity, ↓CSE, ↔AM potency, ↓E&lt;sub&gt;max&lt;/sub&gt;</td>
<td>HEK293</td>
<td>[8]</td>
</tr>
<tr>
<td>C(1-8)</td>
<td>Deletion</td>
<td>CLR</td>
<td>↓AM binding and affinity, ↓CSE, ↔AM potency, ↓E&lt;sub&gt;max&lt;/sub&gt;</td>
<td>HEK293</td>
<td>[8]</td>
</tr>
<tr>
<td>C(1-7)</td>
<td>Deletion</td>
<td>CLR</td>
<td>↔AM binding, ↓AM affinity and potency, ↓E&lt;sub&gt;max&lt;/sub&gt;, ↓CSE</td>
<td>HEK293</td>
<td>[8]</td>
</tr>
<tr>
<td>C(1-6)</td>
<td>Deletion</td>
<td>CLR</td>
<td>↔AM binding, ↓AM affinity and potency, ↓E&lt;sub&gt;max&lt;/sub&gt;, ↓CSE</td>
<td>HEK293</td>
<td>[8]</td>
</tr>
<tr>
<td>C(1-3)</td>
<td>Deletion</td>
<td>CLR</td>
<td>↓AM binding, ↓AM affinity and potency, ↓E&lt;sub&gt;max&lt;/sub&gt;, ↓CSE</td>
<td>HEK293</td>
<td>[8]</td>
</tr>
<tr>
<td>C</td>
<td>Chimaeras: CRR of R1 and R3</td>
<td>CLR</td>
<td>↔CSE</td>
<td>HEK293</td>
<td>[8]</td>
</tr>
<tr>
<td>C(1-9)</td>
<td>Deletion</td>
<td>CLR</td>
<td>Cos 7 cells: ↔hAM and hαCGRP potency, HEK293 cells: ↑hAM potency, ↔hαCGRP potency</td>
<td>Cos 7 and HEK293</td>
<td>[7]</td>
</tr>
<tr>
<td>C(1-9)</td>
<td>Deletion</td>
<td>CT&lt;sub&gt;(a)&lt;/sub&gt;</td>
<td>Poor AMY&lt;sub&gt;2(a)&lt;/sub&gt; receptor phenotype, ↓rAmy binding, ↓rAmy and hCGRP binding, ↔hαCGRP, rAmy and sCT potency, ↓hCT potency</td>
<td>Cos 7</td>
<td>[7]</td>
</tr>
<tr>
<td>C(1-4)</td>
<td>Chimaera: CRR of R3</td>
<td>CLR</td>
<td>↑recycling, ↑resensitisation, ↔CSE</td>
<td>HEK293</td>
<td>[9]</td>
</tr>
</tbody>
</table>
1.9.4.3 RAMP3

Like RAMP2, there is only limited mutagenesis data available for RAMP3. The mutagenesis data generated in the RAMP3 N terminus from the literature are summarised in Table 1.4.

1.9.4.3.1 Predicted helix 1 with CLR; AM2 receptor

The N-terminal cysteine residues have been examined in both mouse and human RAMP3. When mouse RAMP3 was cotransfected with CLR in xenopus oocytes, individual mutations of each cysteine to serine mostly led to reduced cell-surface expression (Flahaut et al., 2003), suggesting that some structural perturbation may have been introduced to the receptor complex by the mutations. In human RAMP3, C40W mutation almost abolished cell-surface expression of the AM2 receptor accompanied with significantly reduced AM response (Bailey et al., 2010). The effect seen with C40W mutation was more detrimental than the alanine substitutions at the equivalent cysteine residues in human RAMPs 1 and 2 (Kuwasako et al., 2003b; Simms et al., 2006) or in mouse RAMP3 (Flahaut et al., 2003). C40 is a conserved cysteine residue in RAMPs that is presumably to form a disulphide bond with C72, however, the modelling of RAMP3 has suggested that simple disruption of this bond is not likely to cause any large perturbation to change the protein folding (Bailey et al., 2010). It seems that the bulky tryptophan substitution might have disrupted either the overall protein structure or interactions with CLR upon the breakage of the disulphide bond and thus the receptor expression and function was severely impaired.

1.9.4.3.2 Predicted helix 2 with CLR; AM2 receptor

The equivalent residues to 86-92 in human RAMP2 were also investigated in human RAMP3 (59-65) (Kuwasako et al., 2001). Deletion of residues 59-65 of human RAMP3 reduced AM potency and diminished specific AM binding when coexpressed with CLR. However, whether this effect seen was due to altered cell-surface expression was unclear. The equivalent region in rat RAMP3 (58-64) has also been characterised (Kuwasako et al., 2002). Deletion of these 7 residues led to a significant reduction in both AM binding and potency at the AM2 receptor, but cell-surface expression was not affected.

SDM has also been conducted to determine the role of individual residues. Position 74 stands out from these studies. W74 in RAMP1 has been demonstrated to be important for the high affinity and selectivity of BIBN4096BS for the human CGRP receptor; the equivalent residue E74 in RAMP3 also plays an important role in ligand binding. Very interestingly, E74W
AM$_2$ displayed a reduced AM potency in the absence of any change in CGRP potency (Hay et al., 2006a).

1.9.4.3.3 Predicted helix 3 with CLR; AM$_2$ receptor

SDM has been performed at two extracellular histidine residues in the predicted helix 3 (Kuwakako et al., 2008). Alanine substitution at H110 did not affect the receptor function or expression when coexpressed with CLR. On the other hand, H97 which is the equivalent residue to H97 in RAMP1 could potentially be involved in forming the CLR binding interface. Consistent with this hypothesis, H97A caused a reduction in both AM binding and potency. However, the cell surface expression was difficult to interpret, as the V5 tagged WT RAMP3 showed significant cell-surface expression in the absence of CLR (Kuwakako et al., 2008). It can be speculated that cotransfection of H97A RAMP3 may have reduced CLR translocation to the cell surface.

1.9.4.3.4 RAMP3/CTR; AMY$_3$ receptor

Like the other two RAMPs, mutagenesis data available with CTR are limited (Table 1.4). Only two studies have been published to date. E74W RAMP3 mutant was characterised with CT$_{(a)}$ in Cos 7 cells and both rAmy and βCGRP potencies were reduced (Hay et al., 2006a). In the other study, the C40W and F100S mutations in RAMP3 induced a reduced rAmy potency whereas the peptide potency was not affected by the W56R and L147P mutations (Bailey et al., 2010).
**Table 1.4** Summary of mutagenesis data that are available in human RAMP3 from the literature. Mutants generated in various studies are grouped based on the positions of the mutations in the RAMP3 model. CRR: corresponding region; R1: hRAMP1; R2: hRAMP2; CSE: cell-surface expression. SDM: site directed mutagenesis; C: C terminus; TpM: triple mutant; $E_{\text{max}}$: maximum response (not significantly changed unless specified in the table); ↔: unchanged compared to WT; ↓: reduced compared to WT; ↑: increased compared to WT; ↓↓: reduced to basal level; ≈: equivalent to. [1]: Bailey et al., 2010, [2]: Kuwasako et al., 2001, [3]: Hay et al., 2006a, [4]: Qi et al., 2008. [5]: Kuwasako et al., 2008, [6]: Kuwasako et al., 2006, [7]: Udawela et al., 2006b, [8]: Bomberger et al., 2005.

<table>
<thead>
<tr>
<th>Position</th>
<th>Mutation</th>
<th>Receptor</th>
<th>Effect</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
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<tr>
<td><strong>Helix 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C40</td>
<td>SDM: C40W</td>
<td>CLR</td>
<td>↓↓hAM potency, ↓↓$E_{\text{max}}$, ↓↓CSE</td>
<td>Cos 7</td>
<td>[1]</td>
</tr>
<tr>
<td>C40</td>
<td>SDM: C40W</td>
<td>CT&lt;sub&gt;(a)&lt;/sub&gt;</td>
<td>↓rAmy potency</td>
<td>Cos 7</td>
<td>[1]</td>
</tr>
<tr>
<td>W56</td>
<td>SDM: W56R</td>
<td>CLR</td>
<td>↔hAM potency, ↔CSE</td>
<td>Cos 7</td>
<td>[1]</td>
</tr>
<tr>
<td>W56</td>
<td>SDM: W56R</td>
<td>CT&lt;sub&gt;(a)&lt;/sub&gt;</td>
<td>↔rAmy potency</td>
<td>Cos 7</td>
<td>[1]</td>
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<td><strong>Helix 2</strong></td>
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<tr>
<td>59-65 Deletion</td>
<td></td>
<td>CLR</td>
<td>↓↓hAM response, ↓↓hAM affinity</td>
<td>HEK293</td>
<td>[2]</td>
</tr>
<tr>
<td>E74</td>
<td>SDM: E74W</td>
<td>CLR</td>
<td>↓hAM potency</td>
<td>Cos 7</td>
<td>[3]</td>
</tr>
<tr>
<td>E74</td>
<td>SDM: E74W</td>
<td>CT&lt;sub&gt;(a)&lt;/sub&gt;</td>
<td>↓rAmy potency, ↓hβCGRP potency</td>
<td>Cos 7</td>
<td>[3]</td>
</tr>
<tr>
<td>E74</td>
<td>SDM: E74Q</td>
<td>CLR</td>
<td>↓hAM potency</td>
<td>Cos 7</td>
<td>[3]</td>
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<tr>
<td>E74</td>
<td>SDM: E74K</td>
<td>CLR</td>
<td>↓hAM potency</td>
<td>Cos 7</td>
<td>[3]</td>
</tr>
<tr>
<td>E74</td>
<td>SDM: E74Q</td>
<td>CLR</td>
<td>↔hαCGRP potency, ↔CSE</td>
<td>Cos 7</td>
<td>[4]</td>
</tr>
<tr>
<td>E74</td>
<td>SDM: E74K</td>
<td>CLR</td>
<td>↔hαCGRP potency, ↓CSE</td>
<td>Cos 7</td>
<td>[4]</td>
</tr>
<tr>
<td><strong>Helix 3</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>H97</td>
<td>SDM: H97A</td>
<td>CLR</td>
<td>↓↓hAM potency, ↓↓specific hAM binding, ↔CSE (WT receptor had significant CSE)</td>
<td>HEK293</td>
<td>[5]</td>
</tr>
<tr>
<td><strong>F100</strong></td>
<td><strong>SDM: F100S</strong></td>
<td><strong>CLR</strong></td>
<td>↔hAM potency, ↓↓E(_{\text{max}}) ↓↓CSE</td>
<td><strong>Cos 7</strong></td>
<td>[1]</td>
</tr>
<tr>
<td><strong>F100</strong></td>
<td><strong>SDM: F100S</strong></td>
<td><strong>CT(_{(a)})</strong></td>
<td>↓rAmy potency</td>
<td><strong>Cos 7</strong></td>
<td>[1]</td>
</tr>
<tr>
<td><strong>H110</strong></td>
<td><strong>SDM: H110A</strong></td>
<td><strong>CLR</strong></td>
<td>↔hAM potency, ↔specific hAM binding, ↔CSE (WT receptor had significant CSE)</td>
<td><strong>HEK293</strong></td>
<td>[5]</td>
</tr>
<tr>
<td><strong>C40 + F100 + L147</strong></td>
<td><strong>TpM: C40W + F100S + L147P</strong></td>
<td><strong>CLR</strong></td>
<td>↓hAM potency, ↓↓E(_{\text{max}}) ↓↓CSE</td>
<td><strong>Cos 7</strong></td>
<td>[1]</td>
</tr>
<tr>
<td><strong>C40 + F100 + L147</strong></td>
<td><strong>TpM: C40W + F100S + L147P</strong></td>
<td><strong>CT(_{(a)})</strong></td>
<td>↓rAmy potency</td>
<td><strong>Cos 7</strong></td>
<td>[1]</td>
</tr>
</tbody>
</table>

### The TM and C-terminal domain

| **C** | **Deletion** | **CLR** | ↔AM binding, affinity and potency, ↔CSE | **HEK293** | [6] |
| **C(1-8)** | **Deletion** | **CLR** | ↔AM binding, affinity and potency, ↔CSE | **HEK293** | [6] |
| **C(1-7)** | **Deletion** | **CLR** | ↔AM binding, affinity and potency, ↔CSE | **HEK293** | [6] |
| **C(1-6)** | **Deletion** | **CLR** | ↔AM binding, affinity and potency, ↔CSE | **HEK293** | [6] |
| **C(1-5)** | **Deletion** | **CLR** | ↔AM binding, affinity and potency, ↔CSE | **HEK293** | [6] |
| **C** | **Chimaera: CRR of R3** | **CLR** | ↔CSE | **HEK293** | [6] |
| **C(1-9)** | **Deletion** | **CLR** | Cos 7 cells: ↔hAM and h\(\alpha\)CGRP potency. HEK293 cells: ↑h\(\alpha\)CGRP potency, ↔hAM potency | **Cos 7 and HEK293** | [7] |
| **C(1-9)** | **Deletion** | **CT\(_{(a)}\)** | ↓rAmy binding, ↔h\(\alpha\)CGRP, rAmy and sCT binding, ↑hCT binding ↓h\(\alpha\)CGRP and rAmy potency, ↑hCT potency, ↔sCT potency | **Cos 7** | [7] |
| **C(1-4)** | **Deletion** | **CLR** | ↓recycling, ↓resensitisation, ↔CSE | **HEK293** | [8] |
| **D145** | **SDM: T145A** | **CLR** | ↓recycling, ↓resensitisation, ↔CSE | **HEK293** | [8] |
| **T146** | **SDM: T146A** | **CLR** | ↓recycling, ↓resensitisation, ↔CSE | **HEK293** | [8] |
| **L147** | **SDM: L147A** | **CLR** | ↓recycling, ↔resensitisation, ↔CSE | **HEK293** | [8] |
| **L147** | **SDM: L147P** | **CLR** | ↔hAM potency, ↔CSE | **Cos 7** | [1] |
| **L147** | **SDM: L147P** | **CT\(_{(a)}\)** | ↓rAmy potency | **Cos 7** | [1] |
| **L148** | **SDM: L148A** | **CLR** | ↓recycling, ↓resensitisation, ↔CSE | **HEK293** | [8] |
1.10 Aims and Objectives

The ligand to receptor interaction is more complicated for the calcitonin family peptides and their receptors than for other family B GPCRs due to the presence of RAMPs. As the N terminus of RAMP appears to contribute to the ligand binding, in addition to that of the receptor itself, the binding mechanism suggested by the two domain model for family B GPCRs must be somehow altered by the presence of RAMPs. There is evidence for RAMP to contribute to the step 1 of the two domain model as the C-terminal residues of AM have been demonstrated to be involved in the interaction with an N-terminal residue of RAMP3 in the AM₂ receptor (Robinson et al., 2009).

Despite the considerable amount of mutagenesis data available for the N-terminal domains of RAMPs and CLR, there is still no clear picture of the structure-function relationship for the RAMP or receptor residues in the calcitonin family receptors. Furthermore, most mutagenesis studies have been focused on the RAMP/CLR complexes; there is little information about the residues in either RAMP or CTR involved in the ligand interactions with the CT or Amy receptors. To generate drugs that target these receptors, a clearer understanding of the role of RAMP and receptor in peptide binding is needed.

Therefore, this thesis aimed to identify key residues or regions in the receptors that are involved in docking CT, CGRPs, Amy and AM by performing a structure-function analysis of residues in the N-terminal domains of the calcitonin family receptors. The studies carried out in this thesis could contribute to understanding the role of RAMP in the ligand-receptor interactions and potentially provide valuable insight to the regions or residues that might be important for these interactions. Such information will not only contribute to the structural determination of the receptor proteins, but also provide specific targeting points that are potentially of great value to drug design. The project has the following specific objectives:

1. Characterisation of RAMP1/3 chimaeric mutants (with CTₐ), Chapter 3.

In this thesis, small chimaeric RAMP1 mutants with every four consecutive residues replaced by the corresponding residues from the N terminus of RAMP3 were characterised with CTₐ. This study aimed at performing a screen of the N terminus of RAMP1 to identify some regions that might be important for the interactions with ligand and receptor.
2. Characterisation of RAMP1 and RAMP3 mutants with amino acid substitutions (with CLR and CT(a)), Chapters 4 and 5.

Strategic substitutions were employed in this study. Residues or regions that are conserved between RAMPs 2 and 3, but different in RAMP1 were swapped between RAMPs 1 and 3. The mutants were characterised with both CLR and CT(a). It was hypothesised that these residues may provide insight into the unique pharmacological properties that RAMP1 or RAMP3 confer.

3. Characterisation of RAMP1 and RAMP3 mutants with amino acid substitutions at position 74 (with CLR), Chapter 6.

Strategic substitutions performed in RAMPs 1 and 3 demonstrated the importance of position 74 to AM potency. Additional substitutions were performed at position 74 in RAMPs 1 and 3 to elucidate the nature of interaction involved at this position contributing to the AM potency.

4. Characterisation of a naturally occurring hCT(a) variant (with and without RAMP1), Chapter 7.

A naturally occurring hCT(a) variant which lacks the first 47 amino acid in its N terminus has been identified (Δ(1-47)hCT(a)) in various human tissues (Albrandt et al., 1995). This deletion includes the predicted signal sequence and one of the four potential N-linked glycosylation sites. Nevertheless, Albrandt et al. reported that the truncated variant was able to respond to hCT and sCT with high potency (Albrandt et al., 1995). On the other hand, the human Amy receptor phenotypes that could potentially result from the interaction between Δ(1-47)hCT(a) and RAMPs have not been described. Interestingly, amino acids 23-60 in the N terminus of CLR have been suggested to mediate its association with RAMP1 (Ittner et al., 2005). This work aimed at performing a thorough characterisation of Δ(1-47)hCT(a) both with and without RAMP1 to determine the role of truncated region in the ligand interaction and CT(a)/RAMP1 association.
Chapter 2
Materials and Methods

2.1 Materials

2.1.1 Peptides

hαCGRP, hβCGRP, hAM2 (47 amino acids), hAM, Tyr° hαCGRP, hCGRP8-37, AC187, hCT, sCT, sCT8-32 and rAmy (rAmy is used in this thesis as hAmy has a strong tendency to aggregate into fibrils in vitro (Goldsbury et al., 1999; Goldsbury et al., 1997) were purchased from American Peptide (Sunnyvale, CA, USA) or Bachem (Bubendorf, Switzerland). The 15-52 fragment of hAM (AM15-52) was kindly provided by Professor David Coy (Tulane University Medical School, New Orleans).

Peptides were purchased as lyophilised preparations and were rehydrated in water for use. The peptide molecular weight and content provided by manufacturers (80% was assumed as peptide content if the information was not available) were used to make 1 mM stock solutions which were stored as aliquots for single use in siliconised microcentrifuge tubes (Bio Plas Inc., San Rafael, CA, USA) at -30 °C.

\(^{125}\)I-hAM\(_{13-52}\) (0.1 mL, 370 kBq, 10 μCi) was purchased from Perkin Elmer (Boston, MA, USA). The peptide was in solution containing trifluoracetic acid/bovine serum albumin (BSA)/1-propanol/acetonitrile, stored at -20 °C.

2.1.2 Reagents

All general chemical reagents were of analytical or equivalent grades, and are not listed for brevity.

- 3':5'-cyclic AMP dependent protein kinase (PKA): Sigma, Cat # P5511
- Activated charcoal: Sigma Cat # C-3345, 100-400 mesh
- Ampicillin: Sigma Cat # A0166-5G
- Bovine serum albumin (BSA): ICP Bio, Cat # ABRE-100G
- Cresyl violet acetate: Sigma, Cat # C1791-1G
- Distilled DNAse/RNAse free water: Gibco, Cat # 10977-015
- DL-Dithiothreitol (DTT) for electrophoresis Cat # D9163-1G
- dNTPs: Promega, Cat # U120/121/122/123B
Chapter 2

- **Dpn I** restriction enzyme: Promega Cat # R6231
- Forskolin: Tocris Bioscience Cat # 1099
- Isobutylmethylxanthine (IBMX): Sigma Cat # 15879
- o-Phenylenediamine dihydrochloride (OPD): SIGMAFAST™ OPD, Sigma, Cat # P9187
- *Pfu* DNA polymerase: Promega Cat # M7741
- Protease inhibitor cocktail for use in mammalian cell and tissue extracts in DMSO solution: Sigma P8340
- Scintillation fluid optiPhase ‘superMix’: PerkinElmer Life & Analytical Sciences B.V., Cat # 1200-439
- Tritiated cAMP [³H] Adenosine 3':5’-cyclic phosphate, ammonium salt (0.74-1.1 TBq/mmol, 37 MBq/ml): Amersham Cat # TRK 304.

2.1.3 Inorganic solutions

- HEPES buffer solution, 1M, pH 7.4: Gibco Cat # 15630
- 1 x Phosphate-buffered saline (PBS): diluted from 10 x PBS solution purchased from Roche, pH7.0

2.1.4 Antibodies

- Anti-c-myc (Ab-1) mouse mAb (9E10): Calbiochem, Cat # OP10
- Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse: Abcam, Cat # 6C5, AB8245
- ECL™ anti-mouse IgG, horseradish peroxidase linked whole antibody (from sheep): Amersham, Cat # NA931V
- Monoclonal MAb 9B4 (IgG2A), mouse anti-human CTR N terminus antibody: Welcome Receptor Antibodies Pty Ltd
- Purified antibody monoclonal HA: COVANCE, Cat # MMS-101P

2.1.5 Cell culture reagents

- Complete growth media: Dulbecco’s Modified Eagle’s Medium (DMEM) high glucose with 0.11g/l Na Pyr and L-glutamine (Gibco, Cat # 11995-065, 500 mL). 40 mL heat-inactivated FBS (8% final, FBS was heat inactivated at 56 °C for 30 minutes) and penicillin/streptomycin antibiotics (5% v/v) were added before use.
- PBS (calcium and magnesium free, Gibco Cat # 14190-144)
2.1.6 Transfection reagents

- TrypLE™ Express: Gibco, Cat # 12605
- Glucose (dextrose monohydrate, Aldrich, Cat # 1-1910)
- Polyethylenimine (PEI, Aldrich 40872-7, 25kD) (Refer to Section 2.2.4 for how to make up working solution)

2.1.7 DNA constructs

2.1.7.1 RAMP constructs

Human RAMP1 with (mycRAMP1) or without a N-terminal myc tag and human RAMP3 were kindly provided by Steven Foord (Glaxosmithkline, Stevenage, UK). Untagged RAMP1 was used in Chapter 3 and mycRAMP1 was used in Chapters 4, 5, 6 and 7. mycRAMP1 has been shown to exhibit equivalent behaviour to the untagged RAMP1 (McLatchie et al., 1998). On the other hand, epitope tagging of human RAMP3 led to significant expression at the cell surface on its own when compared to untagged hRAMP3 (Christopoulos et al., 2003; Kuwasako et al., 2008). Therefore, untagged RAMP3 was used in this thesis.

The 26 RAMP1/3 chimaeras (every four residues in the N terminus of human RAMP1 replaced by the corresponding regions from human RAMP3) were made in untagged human RAMP1 by Dr John Simms (Aston University, Birmingham, United Kingdom). In addition, myc tagged version of three chimaeras, RAMP338-41, RAMP362-65 and RAMP390-93 were made in mycRAMP1 in this thesis for total cellular expression analysis using western blotting. The reciprocal mutant RAMP186-89 to RAMP386-89 was made in untagged RAMP3 in this thesis.

All the RAMP1 mutants characterised in Chapters 4, 5 and 6 were made in mycRAMP1 and the RAMP3 mutants were made in untagged RAMP3 in this thesis.

2.1.7.2 CLR constructs

Human CLR with a N-terminal double hemagglutinin (HA) epitope tag (HA-CLR) was kindly provided by Steven Foord (Glaxosmithkline, Stevenage, UK).
2.1.7.3 CTR constructs

The insert negative form of human CTR (CT\textsubscript{(a)}) with the leucine polymorphic variant at position 447 (Pham et al., 2004) was used in this thesis. CT\textsubscript{(a)} (leucine polymorphic variant) with a N-terminal double HA epitope tag (HA-CT\textsubscript{(a)}) was provided by Prof Patrick Sexton (Monash University, Melbourne, Australia). Untagged CT\textsubscript{(a)} (leucine polymorphic variant) was kindly provided by Dr Sebastian Furness (Monash University, Melbourne, Australia). The human CT\textsubscript{(a)} mutant with a truncation of 1-47 amino acids at the N terminus (proline polymorphic variant) was provided by Prof Laurence Miller (Mayo clinic, Scottsdale, Arizona, USA).

2.1.8 Mammalian cells

Cos 7 cells were used for studying the calcitonin family receptors in this thesis as they have been shown to lack significant levels of endogenous RAMPs, CLR and CTRs (Bailey & Hay, 2006; Conner et al., 2005). The receptor components that were transfected into these cells can thus been accurately compared without the interference from any significant background expression of these proteins. The cells were kindly donated by Dr Nigel Birch (School of Biological Sciences, the University of Auckland) and have been routinely used for studying the calcitonin-family peptide receptors in our laboratory.

2.2 Methods

2.2.1 Site-directed mutagenesis

Site-directed mutagenesis was performed using an in-house version of the Stratagene QuickChange method (Bailey & Hay, 2007). The desired mutations were introduced into the double-stranded plasmid DNA sequence using a primer pair complementary to opposite strands of the DNA insert in the vector. The primers were extended and mutated plasmid was amplified during temperature cycling by Pfu DNA polymerase. Dpn I endonuclease, which targets methylated and hemimethylated DNA (sequence recognised: 5´-G\textsuperscript{me}ATC-3´ and 3´-CT\textsuperscript{me}AG-5´ at the opposite strand), was then used to digest the parental DNA template. The nicked plasmid was transformed into competent cells, where the nick was repaired. Plasmid was isolated and the mutation was checked by sequencing (See Section 2.2.1.3 and 2.2.1.4).
2.2.1.1 Primer Design

Forward and reverse primers were designed from nucleotide sequences of the receptor components with relevant base changes to incorporate amino acid point mutations. The primers were designed using the following criteria:

- Forward and reverse primers anneal to the same sequence on opposite strands of the plasmid.
- Primers are between 25 and 45 bases in length, with a melting temperature of $\geq 78^\circ C$ (formula used for calculating melting temperature: $T_m = 81.5 + 0.41(%\text{GC}) - 675/\text{primer length} - %\text{mismatch}$).
- The desired mutation point(s) is in the middle of the primer with ~10–15 bases of correct sequence on both sides.
- The primers have $\geq 40\%$ GC content and end with one or more C or G bases.

All primers (standard desalted) were custom made by Invitrogen or Integrated DNA technologies (Coralville, USA). Primers were rehydrated in water to make up 100 μM stock solutions and stored at -20 °C. The oligonucleotide sequences of the primers used in this thesis are listed in Appendix.

2.2.1.2 Mutagenesis

1 μL template DNA (~100ng), 1 μL of forward and reverse primer mix (5 pmol/μL each), 5 μL Pfu DNA Polymerase 10x Reaction Buffer with MgSO₄, 1 μL dNTPs mix (10 mM each dNTP), 1 μL Pfu DNA polymerase and 41 μL water were mixed in a PCR tube. The reaction mix then underwent thermal cycling in a thermocycler (Eppendorf) following the steps as 1) Denaturation at 95 °C for 30 seconds; 2) Denaturation at 95 °C for 30 seconds; 3) Annealing at 55 °C for 60 seconds; 4) Extension at 68 °C for 530 seconds (120s/kb); 5) cycling from 2) to 4) for 12 times (single base changes), 16 times (single amino acid changes) or 18 times (multiple amino acid changes). Following the thermal cycling, the reaction mix was cooled on ice and briefly centrifuged; 1-2 μL Dpn I was added to the reaction mix which was subsequently heated at 37 °C for 1-2 hours.

2.2.1.3 Transformation

The mutated plasmids were transformed into XL-Gold® Ultracompetent cells (Stratagene, Cat # 200315). 50 μL cells were first thawed on ice and then incubated with 2 μL β-
mercaptopoethanol for 10 minutes on ice (with gentle mixing every 2 minutes). 2 μL of PCR products were subsequently added to the cells and incubated on ice for 30 minutes. Cells were heat-shocked at 42 ºC for exactly 30 seconds, and returned back on ice for 2 minutes. Cells were then spread onto Luria Broth agar plates containing ampicillin (0.1 mg/mL as final concentration) and grown at 37 ºC for 12-16 hours.

2.2.1.4 Isolation of plasmid for sequencing

The mutated plasmid was amplified and isolated to verify the sequence. Two methods were employed in this thesis: Wizard® Plus SV Minipreps DNA purification system (Promega Cat # 1330) and Templiphi 100 Amplification Kit (GE healthcare, Cat # 25-6400-10).

When the Wizard® Plus SV Minipreps DNA purification system was used, two colonies were picked from each transformation plate and grown independently in 5 mL Luria broth containing ampicillin (0.1 mg/mL as final concentration) for ~16 hours at 37 ºC with shaking. The overnight cultures were pelleted in a centrifuge (1660 x g, 5-15 minutes, 4 ºC). Plasmid DNA was isolated from the cells following the steps recommended in the manufacturer’s instructions for the Wizard® Plus SV Minipreps DNA purification system. Briefly, the cell pellet was resuspended, lysed, treated with alkaline protease to inactivate endonucleases and other proteins that may affect the quality of the DNA isolated. The cell lysate was then neutralised and pelleted to remove cell debris. The supernatant was subsequently loaded onto the spin column and centrifuged. After two washing steps, DNA was collected in 50 μL distilled DNAse/RNAse free water. Isolated plasmid DNA was sequenced (by SBS sequencing facility) to verify the introduction of the desired mutations (vector specific primers were used).

In the later part of this thesis, plasmids were isolated and amplified in using Templiphi Amplification Kit. A small portion of colonies (“needle touch” of pipette tip) was incubated in 5 μL Sample Buffer provided in the kit at 95 ºC for 3 minutes in a PCR machine. Two colonies from each transformation plate were routinely selected and used for Templiphi reactions. The leftovers of the colonies selected were marked on the transformation plate and each of the Templiphi reaction tubes was labelled in accordance with the colony that they contained. The lysed cells in the buffer were cooled on ice and incubated with 5 μL reaction buffer and 0.2 μL enzyme mix at 30 ºC for 7 hours (longer incubation allows inhibited reaction to use up all deoxynucleotides present). The enzymes were lastly inactivated at 65 ºC for 10 minutes. The amplified plasmid DNA was diluted in ~25 μL water for sequencing.
2.2.2 Maxiprep DNA preparations

2.2.2.1 Maxiprep DNA preparations from SDM products

After the mutated plasmid DNA sequences were verified by sequencing, the DNA was amplified and isolated on a larger scale for use in cell transfection. In the case of the Wizard® Plus SV Minipreps DNA purification system, an additional transformation step was performed, where 1 μL of diluted miniprep DNA (~10 ng) was transformed into 9 μL XL-Gold® Ultracompetent cells (30 minutes incubation on ice, heat-shock at 42 °C for 30 seconds followed by 2 minutes incubation on ice). The transformed cells were spread on a Luria Broth agar plate containing ampicillin and the plate was incubated at 37 °C for 12-16 hours. DNA was isolated from a 200 mL overnight culture of one colony from this transformation plate using Genomed Jetstar Maxiprep Kit (Astral Scientific, Cat # 220020) or PureLink™ HiPure Plasmid Filter Purification Kits (Invitrogen, Cat # K210017). Maxiprep DNA isolation was carried out according to the manufacturer’s instructions, except that no ethanol precipitation was performed and the DNA pellet obtained was re-dissolved in 500-650 μL RNAse/DNase free water. Concentration and purity (the plasmid DNA that had a 260/280 ratio between 1.80 and 2.00 was used) of the plasmid DNA isolated was determined using NanoDrop™ machine. A small portion of DNA was then diluted and sequenced.

When the Templiphi reaction was performed, no additional transformation step was required. Large scale isolation of the mutated plasmid DNA was performed using an overnight culture of the colony from which plasmid sequence had been confirmed to contain the right mutation in the sequencing reactions, as the majority of the colony was left untouched on the plate.

2.2.2.2 Maxiprep DNA preparations from existing maxiprep DNAs

Maxiprep DNAs were also prepared from the existing preps of which sequences had been verified. These were routinely performed mainly for the WT constructs when the old preps were used up. 1 μL of diluted maxiprep DNA (~10 ng) was transformed into 9 μL XL-Gold® Ultracompetent cells by 30 minutes incubation on ice followed by heat-shock at 42 °C for 30 seconds and then 2 minutes incubation on ice. The transformed cells were spread and grown on a Luria Broth agar plate containing ampicillin overnight. Maxiprep DNA was prepared from the overnight culture of one of the colonies on the transformation plate as described in Section 2.2.2.1 above.
2.2.3 Cell culture

Cos 7 Cells were cultured in T75 cm$^2$ or T175 cm$^2$ culture flasks in complete growth media and kept in a 37 °C humidified 95% air/5% CO$_2$ incubator. To subculture cells grown in T75 cm$^2$ flasks, old medium was first removed from flasks and cells were briefly washed with 5 mL 1×PBS (Ca$^{2+}$, Mg$^{2+}$ free). 5 mL TrypLE was used to detach cells from the plastic, and 5 mL of complete growth media were subsequently added to neutralise the effect of TrypLE. A portion of the cell suspension (1 mL and 2 mL were used for routine 1:10 and 1:5 splits, respectively) was transferred into a new T75 cm$^2$ flask with ~13 mL complete growth media added. For plating out cells for transfection, a portion of cell suspension was diluted with complete growth media in 50 mL falcon tubes (1 part of cell suspension: 4 parts of medium). After gentle mixing, cell suspension was aliquoted into wells of 96 well culture plates (100μL per well) for cAMP assays and ELISAs or 6 well culture plates (2 mL per well) for western blotting experiments. Cells were allowed to grow as monolayers to ~80% confluence in plates for approximately one day prior to transfection. Cos 7 were kept until they reached passage number 30; a new vial of Cos 7 cells was then used from the liquid nitrogen stocks in the laboratory.

For the radioligand binding assays, Cos 7 cells were grown in T175 cm$^2$ flasks. To subculture these cells, 10 mL 1×PBS (Ca$^{2+}$, Mg$^{2+}$ free) was used to wash the cells and 10 mL TrypLE was used to detach cells from the plastic. 10 mL of complete growth media were subsequently added to neutralise the effect of TrypLE. A portion of the cell suspension (2 mL and 4 mL were used for 1:10 and 1:5 splits, respectively) was transferred into a new T175 cm$^2$ flask with ~20 mL complete growth media added.

2.2.4 Transient transfection

Transient transfection was performed using PEI (Boussif et al., 1995). PEI binds to DNA molecules and forms a complex with DNA (polyplex). The polyplex is positively charged and is brought into the cell via endocytosis. The polyplex is then released into the cytoplasm as the vesicle swells and bursts as a result of the changed osmotic potential in the cell. The DNA is free to diffuse into the nucleus when the polyplex unpacks (Akinc et al., 2005).

A stock solution was made by dissolving PEI in water to give 0.9 mg/mL as final concentration which was equivalent to ~10 mM. This molarity was calculated according to the molecular weight of the monomer (Boussif et al., 1995). The pH was corrected to 7.5.
before the volume was adjusted to the final amount with water. The stock PEI solution was filter-sterilised and aliquots were stored at -20 ºC.

The transfection efficiency in this method is dependent on the ratio of phosphate (P) in the DNA to the nitrogen (N) in the PEI (Boussif et al., 1995). There is 3 nmol phosphate in 1μg DNA whereas 10 nmol amine nitrogen is available for protonation in 1 μg of 10 mM PEI solution. The amount of 10 mM PEI solution needed for transfection can be calculated by taking into account the N/P ratio, as below:

\[
\text{Amount of 10 mM PEI solution to be used (μL)} = \frac{\text{μg DNA} \times 3 \times \text{N/P ratio}}{10}
\]

The N/P ratio needs to be determined for each cell line for the transfection efficiency and toxicity for PEI. It has been previously optimised for Cos 7 cells in our laboratory; a value of 10 has been routinely used for Cos 7 cell transfection. The equation above is simplified as below:

\[
\text{Amount of 10 mM PEI solution to be used (μL)} = \text{μg DNA} \times 3
\]

Equal amounts of plasmid DNA for each receptor component i.e. CLR/CTR and RAMP/pcDNA3 vector were used for transfection. 250 ng of plasmid DNA in total was used for transfection per well for 96 well plates, or 5 μg per well for 6 well plates, or 80 μg of plasmid DNA for a T175 cm² flask. The DNA was first diluted in 5% glucose (filter sterilised) (glucose solution was 10% of the total transfection mixture volume) and mixed. The appropriate amount of 10 mM PEI solution was then added to the glucose/DNA mix in a dropwise motion and gently mixed. This transfection mixture was incubated at room temperature for 10 minutes. Following incubation, complete growth media were added to the transfection mix. Old growth medium was aspirated from the cells in plates or flasks and the transfection mix was added in a volume of 100 μL per well for the 96 well plates or 20 mL per T175 cm² flask. Cells were used for experimentation 36-48 hours later.

2.2.5 Cell-based ELISA

Enzyme-Linked ImmunoSorbent Assay (ELISA) was used to measure the cell-surface expression of receptors. Transfected Cos 7 cells in 96 well plates were firstly fixed onto the plastic with 100 μL paraformaldehyde in 1 x PBS (pH 7.4, 4% final concentration in wells) with gentle shaking for 20 minutes at room temperature. After washing of cells in 1 x PBS
twice, 100 μL PBS/0.6% hydrogen peroxide (final concentration in wells) was then added and plates were incubated at room temperature with gentle shaking for 20 minutes. Following one washing step in 1 x PBS, 100 μL PBS/10% goat serum was added to each well and incubated at room temperature with gentle shaking for 1 hour. This mixture was subsequently replaced with 50 μL primary antibody (diluted in PBS/1% goat serum: 1:250 for anti-myc antibody; 1:2000 for anti-HA antibody; 1:500 for MAb 9B4 antibody) and the plates were incubated at 37°C for 30 minutes. Following a brief wash in 1 x PBS, 50 μL secondary antibody (1:500 dilution in PBS/1% goat serum) was added to each well and incubated at room temperature with gentle shaking for 1 hour. The cells were then washed twice with 1 x PBS after which 50 μL OPD solution (one tablet of OPD and one tablet of buffer with urea dissolved in 20 mL water) was added to each well and incubated at room temperature with gentle shaking in the dark for exactly 15 minutes. The reaction was then stopped by adding 50 μL of 0.5 M H₂SO₄ to each well; absorbance was read at 490 nm and 650 nm on a plate reader (Spectra MAX 340, Molecular Devices Corp. or Envision 2104 Multilabel Reader, Perkin Elmer).

Cell density was further determined with cresyl violet staining, where 50 μL working cresyl violet solution (9 parts acid solution (0.6% v/v glacial acetic acid solution), 1 part basic solution (0.1 M sodium acetate), 0.25 part 1% filtered aqueous cresyl violet solution) was added to each well and incubated at room temperature with gentle shaking for 30 minutes. Following a washing step in 1xPBS for 10 minutes, 100 μL 1% SDS was added to each well, shaking for an hour at room temperature to solubilise the cells. The absorbance was read at 595 nm on a plate reader.

Raw data for each well was calculated by subtracting the absorbance reading at 650 nm from the reading at 490 nm and then divided by the reading at 595 nm. The raw data were then normalised to the appropriate WT expression levels as 100% and vector controls (cells transfected with vector alone) as 0%.

2.2.5.1 Cell-surface expression measured by mycRAMP1 vs HA-CLR

Neither CLR nor RAMP can be efficiently translocated and expressed at the cell surface on its own, therefore measuring the HA-CLR or mycRAMP1 expression can be used as an indication of the CGRP receptor complex formation at the cell surface. Figure 2.1 shows the cell-surface expression of some CGRP receptors estimated by measuring HA-CLR (a) and mycRAMP1 (b) expression. Despite the slight variations in the absolute values for each
individual receptor complex, the expression data measured by HA-CLR and mycRAMP1 are consistent. The cell-surface expression of all the mutant CGRP receptors was not significantly different from the WT CGRP receptor in both cases (one-way ANOVA followed by Dunnett’s test).

![Figure 2.1](image)

**Figure 2.1** Cell-surface expression of CGRP receptors containing mycRAMP1 mutants W74E, W74Y, W74A, W74N and WT in complex with HA-CLR, measuring HA-CLR (a) and mycRAMP1 (b) expression. Data are mean ± s.e.m. of three independent experiments, each performed with four replicates. Data were analysed by one-way ANOVA followed by Dunnett’s test.

All RAMP3 constructs made in this thesis were not tagged, thus the expression of the CLR/RAMP3 complexes could only be estimated by measuring HA-CLR expression at the cell surface (Chapter 5). To be consistent with the CLR/RAMP3 complexes, expression of all CLR/mycRAMP1 complexes was also determined by measuring HA-CLR expression (Chapters 4 and 6) in this thesis. In the case of determining AMY1(a) receptor expression, mycRAMP1 expression was measured (Chapter 7) as CT(a) can be expressed and acts as an CT receptor on its own thus has substantial expression at the cell surface (Figure 2.2 and 2.3).

### 2.2.5.2 Determination of anti-CTR antibody (9B4) specificity

Anti-CTR antibody 9B4 was a gift provided by Dr Peter Wookey from Welcome Receptor Antibodies Pty Ltd. Unlike anti-HA and anti-myc antibodies which recognise an epitope of the tagged region in the protein, 9B4 recognises the native CTR residues beyond residue 47. It is very important to demonstrate the specificity of this antibody as GPCR antibodies are
frequently non-specific. The optimal antibody dilution (1:500) which gave the highest signal
to background ratio in ELISA experiments was first determined (data not shown) and
antibody specificity was tested against cells expressing CT(a) and CLR constructs (Figure 2.2).
Cell-surface expression determined using 9B4 showed that CT(a) was expressed both in the
presence and absence of RAMP1 at levels substantially higher than that from the vector
transfectant. In addition, the expression of CT(a) with a 1-47 truncation was not different to
the full length CT(a), agreeing with the antibody epitope position locating beyond residue 47
which was informed by Welcome Receptor Antibodies Pty Ltd. On the other hand, CLR
showed a comparable expression level to the vector transfectant, indicating that 9B4 does not
cross-react with this closely related protein.

![Graph showing cell-surface expression of CT constructs and CLR measured using 9B4.](image)

**Figure 2.2** Cell-surface expression of CT(a) constructs and CLR measured using 9B4. The
graph is representative of three experiments. Data shown are ratios of absorbance readings
from ELISA, performed with three replicates.

In addition, the cell-surface expression measured by 9B4 and anti-HA antibodies were
compared for a mutant (P248A HA-CT(a)) which has been previously reported to have
reduced cell-surface expression compared to the WT receptor (Bailey & Hay, 2007) (Figure
2.3). Figure 2.3a shows that absorbance ratio for HA-CT(a) measured by 9B4 was slightly
lower but still comparable to that measured by anti-HA antibody. The expression of mutant
P248A HA-CT(a) measured by 9B4 (Figure 2.3b) and anti-HA (Figure 2.3c) was consistent;
its expression was abolished at the cell surface in both measurements.
Figure 2.3 Comparison of cell-surface expression of HA-CT\(_{(a)}\) constructs measured using 9B4 and anti-HA antibody (a) Cell-surface expression of HA-CT\(_{(a)}\) and vector measured using 9B4 and anti-HA antibodies. Data are ratios of absorbance reading from three to six replicates. (b) & (c) Cell-surface expression of P248A HA-CT\(_{(a)}\) and WT HA-CT\(_{(a)}\) measured using 9B4 and anti-HA antibodies, respectively. Data shown are from six replicates, normalised to the WT (100%) and vector (0%).

2.2.6 Radio-receptor cAMP assay

As described in Sections 1.6.3 and 1.7.3, the G\(_\alpha_\text{s}\)-mediated cAMP response is the best understood major signal transduction pathway for the calcitonin family receptors. Measuring cAMP as an indication of receptor activation has been commonly used to study these receptors in Cos 7 cells (Bailey & Hay, 2007; Christopoulos et al., 1999; Hay et al., 2005; Simms et al., 2009; Simms et al., 2006). Therefore, receptor function was also characterised by measuring intracellular cAMP accumulation in Cos 7 cells in this thesis.

2.2.6.1 Stimulation of cells

Growth medium in the wells of 96 well culture plates was first aspirated and replaced with 50 μL cAMP assay media (DMEM free from serum or antibiotics, containing 0.1% BSA and 1 mM IBMX (dissolved in 0.1 M NaOH) for 30 minutes to minimise basal cAMP levels. 50 μL peptide concentrations ranging from 2 \(\times\) 10\(^{-12}\) to 2 \(\times\) 10\(^{-6}\) M (giving final concentration of peptide ranging from 10\(^{-12}\) to 10\(^{-6}\) M in wells) or forskolin (as a positive control, at a final concentration of 50 μM) or cAMP assay media alone (as a negative control) was added to
each well. The cells were incubated at 37 °C for 15 minutes. Following the incubation, wells contents were thoroughly aspirated; cAMP was extracted by addition of 50 μL ice-cold absolute ethanol and plates were incubated at -20 °C for at least 5 minutes.

### 2.2.6.2 cAMP measurement

The relative quantities of cAMP in each sample were measured using a radio binding protein assay. Ethanol was first evaporated completely from the 96 well plates using a speed-vac machine (Eppendorf, Concentrator 5301), then 30 μL cAMP assay buffer (20mM HEPES, 5mM EDTA, pH 7.5) was added to each well and incubated at room temperature with gentle shaking for at least 5 minutes to rehydrate cell extracts. 25 μL of the cell extracts were transferred to round-bottom 96 well plates to which 25 μL dilute ³H cAMP (2 μL ³H cAMP stock was diluted in 4 mL cAMP assay buffer) was added to each well of the plates before the addition of 50 μL dilute binding protein (1:2 dilution of the stock binding protein solution (0.02% w/v PKA, 1mM Na citrate, 2mM DTT, pH 6.5) in cAMP assay buffer). The plate contents were mixed, sealed and incubated at 4 °C for 2-24 hours. Following the incubation, 50 μL charcoal (5% (w/v) charcoal/0.2% (w/v) BSA, made in cAMP assay buffer) was added to each well to trap unbound ³H cAMP. Plates were mixed and centrifuged at ~1800 x g for 5 minutes at 4 °C to pellet charcoal. 75 μL of the clear supernatant containing ³H-cAMP bound to PKA was carefully transferred to 96 well flexiplates. 200 μL scintillant (Optiphase supermix, PerkinElmer, Cat # 1200-439) was then added to each well of the flexiplates. Plates were sealed, mixed and counted for 1 minute in a beta counter (1450 Microbeta Plus Liquid Scintillation Counter, Perkin Elmer).

### 2.2.7 Membrane preparation

Transfected cells grown in two T175 cm² flasks were harvested and combined for the preparation of crude membranes for radioligand binding assays. Old media were removed from flasks and cells were washed twice with 5 mL ice cold 1 x PBS. A total of 5 mL of membrane harvest buffer (50 mM HEPES, 1 mM EDTA disodium (EDTA-diNa), pH 7.5) with protease inhibitor cocktail (Sigma, 1:100 dilution) was added to the flasks, and cells were scraped off from the plastic using a cell scraper (Greiner Bio). Harvest buffer containing the dislodged cells was then transferred to 15 mL Falcon tubes on ice. The remaining cells were rinsed from the flask with a further 2.5 mL harvest buffer. The cells were subsequently homogenised on ice using an electric homogeniser (Ika–Werke Ultra-Turrax T8) for 4 periods of 15 seconds and centrifuged at 48,000 x g at 4 °C for 1 hour. The protein pellet
obtained was re-suspended in 5 mL harvest buffer and re-homogenised as above. A small aliquot of the protein was taken for the protein content assay and the remainder was frozen at -80 °C for binding assays.

### 2.2.8 Cell lysate preparation

Old media were removed from wells of the 6 well plate and cells in each well were washed twice with 2 mL ice cold 1 x PBS. 1 mL ice cold 1 x PBS was subsequently added and cells were scraped off from the plastic using a cell scraper (Greiner Bio). PBS containing the dislodged cells was then transferred to 1.5 mL eppendorf tubes on ice. The remaining cells were rinsed from the well with a further 0.5 mL ice cold 1 x PBS. The cells were pelleted at 3300 × g in a bench-top microcentrifuge for 5 minutes at 4 °C. The pellets were resuspended in 50 μL cell lysis buffer (1% v/v triton X-100, 10% v/v glycerol, 150mM NaCl, 20mM HEPES, pH 7.5) with protease inhibitor cocktail (Sigma, 1:100 dilution) and incubated for 30 minutes on ice. The lysed cells were then centrifuged at 9300 × g for 20 minutes at 4 °C. The supernatants containing the soluble proteins were kept; a small aliquot was taken for the protein content assay and the remainder was frozen at -80 °C for western blotting analysis.

### 2.2.9 Protein content assay

Protein quantitation of the membrane preparations was carried out using a colorimetric assay based on the method of Bradford (Bradford, 1976) but using a protocol and solution obtained from Bio-Rad (Quick Start Bradford Dye Reagent 1x protein measurement assay, BioRad, Cat # 500-0205). The assay is based on the change in absorbance maximum observed when Coomassie Brilliant Blue G-250 dye reacts with protein. The method recommended in the manufacturer’s instructions for microplate standard assay was adopted in this thesis for quantifying protein content in the membrane preparation and cell lysate samples (cell lysate samples were diluted according to the detergent tolerance for this assay recommended in the manufacturer’s instructions). BSA in concentrations ranging from 2 mg/mL to 0.063 mg/mL (in membrane harvest buffer or diluted cell lysis buffer) was used to construct a standard curve. 5 μL of each BSA concentration, 5 μL membrane harvest buffer (as 0 concentration control) and 5 μL of membrane preparations were loaded in duplicate into a flat-bottomed 96 well clear plate. The standards or samples were incubated with 250 μL of 1 x dye reagent at room temperature for 5 minutes before measuring the absorbance at 595nm in a plate reader (Envision 2104 Multilabel Reader, Perkin Elmer). Protein content was calculated from the standard curve and adjusted by dilution factor where necessary. The average protein
concentration obtained was ~0.8 mg/mL for the membrane preparations and ~1 mg/mL for the cell lysate samples.

2.2.10 $^{125}$I-hAM$_{13-52}$ binding assay

$^{125}$I-hAM$_{13-52}$ binding was performed on the membrane preparations in duplicate in siliconised microcentrifuge tubes (Bio Plas Inc., San Rafael, CA, USA). A dilution series of non-radio-labelled hAM was prepared in binding buffer (20 mM HEPES, 5 mM MgCl$_{2}$·6H$_2$O, 5 mM KCl, 10 mM NaCl, 1 mM EDTA-diNa, pH 7.4, 0.1% BSA), giving final concentrations ranging from $10^{-6}$ (nonspecific binding) to $10^{-11}$. $^{125}$I-hAM$_{13-52}$ was diluted with binding buffer to allow approximately 30,000 count per minute (cpm) in 50 μL (~9.1 pM). Membranes were thawed on ice and re-homogenised with a glass homogeniser if the membranes were not an even suspension. 50 μL diluted radiolabel, 50 μL appropriate unlabelled competitor (hAM) from the dilution series or buffer (total binding) and 76-100 μg membranes were added to each assay tube in this order. The mixture was mixed and incubated at 4 ºC for 30 minutes. The membranes were pelleted in a bench-top microcentrifuge at 4 ºC for 5 minutes. The supernatants were carefully removed and the pellets were washed once with 500 μl ice-cold binding buffer. Supernatants were discarded after centrifugation and pellets were counted for γ–radiation for 1 minute.

2.2.11 Western Blotting

15-20 μg of cell lysates were denatured in 4 x NuPage LDS sample buffer (Invitrogen) containing 200 mM DTT in a heating block at 95ºC for 10 minutes and then separated in NuPAGE 4-12% Bis-Tris precast gel system (Invitrogen) at 200V for 40 minutes. Proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Invitrogen) using the iBlot dry blotting system (Invitrogen) following manufacturer’s instructions. Blots were then blocked with 5% milk in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween20 (TBS-T) for 1 hour at room temperature and incubated overnight at 4ºC with 1:400 dilution of primary mouse anti-myc mAb (9E10, Calbiochem) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at a dilution of 1:200000 (Abcam, 6C5, AB8245) prepared in TBS-T containing 2.5% milk. After three washes the next day, blots were incubated for 1 hour at room temperature with a 1:2000 dilution of secondary ECL sheep anti-mouse horseradish peroxidase linked antibody prepared in 5% milk in TBS-T. Blots were washed and developed with ECL Plus western blotting detection system (Amersham)
for 5 minutes, and the chemiluminescent signal was detected using LAS3000 imaging system (Fujifilm).

2.2.12 Data analysis and statistical procedures

All the data in this thesis were generated from at least three independent experiments unless otherwise stated, each performed in duplicate in the radioligand binding assays, triplicate in the cAMP assays, or three to eight replicates in the ELISAs. Data were analysed using Graphpad Prism (versions 4.02 or 5.01, GraphPad Software Inc., San Diego, CA). All values are quoted as mean ± s.e.m.

cAMP data generated from the radioreceptor assay were normalised to the responses obtained to 50 μM forskolin (100%) and media controls (0%). For agonist responses, data were fitted to obtain concentration response curves using a four-parameter logistic equation (Hay et al., 2005). F-test was conducted to compare if the Hill slope of the curves was significantly different to 1. In most analyses, the Hill slope was not different to 1. Therefore the Hill slope was constrained to a value of 1. pEC$_{50}$ and E$_{max}$ were obtained from the concentration response curves and compared between the mutant and WT receptors using paired or unpaired t-tests; significance was achieved at $p < 0.05$. For some low potency responses, the curves were largely shifted towards right along the agonist concentration scale and no properly defined E$_{max}$ could be assigned to the curves by the PRISM default curve fitting parameters. In these cases, the tops of the curves were fixed to the maximum point. A representative graph that best present the mean values of pEC$_{50}$ and E$_{max}$ and more importantly the fold shifts in these values was chosen from all the individual experiments and is presented in the thesis. Of note, due to the variations between experiments, the values (e.g. pEC$_{50}$, E$_{max}$ and fold shifts) read off from the representative curves do not always agree completely with the mean values shown in the text which were calculated from all experiments.

For calculation of antagonist affinity in cAMP assays, agonist concentration response curves in the absence and presence of antagonist were globally fitted to Schild analysis (Hay et al., 2005). After confirming that the slope was not significantly different to 1, it was constrained to 1. The resulting estimate of pA$_2$ represents the pK$_B$ (Neubig et al., 2003). Unpaired t-tests were used to compare data; significance was achieved at $p < 0.05$. 
In ELISA, expression values were normalised to WT expression levels as 100% and pcDNA3 alone as 0%. All data were compared using one-way ANOVA (one way analysis of variance) followed by Dunnett’s test for the comparisons between the mutant and WT receptors, or Tukey’s test for multiple comparisons. Significance was achieved at \( p < 0.05 \).

For the binding assay, \(^{125}\text{I}-\text{hAM}_{13-52}\) binding data (cpm) were first converted to fmol radiolabel bound per mg membrane protein using the formula below:

\[
\frac{\text{cpm}}{2.2 \times 10^{12} \times \text{specific activity of radioligand} \times 10^{15} \times \frac{1000}{\text{protein (\(\mu\)g)}}}
\]

The specific activity of \(^{125}\text{I}-\text{hAM}_{13-52}\) used was \(2.2 \times 10^6\) Ci/mol and 76-100 \(\mu\)g of membrane proteins were used in the binding assays. Specific binding (fmol mg\(^{-1}\)) was calculated for each data point by subtracting the mean value obtained using the above formula at the top concentration of hAM from the value of each replicate at different concentration points. Data were fitted to obtain pIC\(_{50}\) values. IC\(_{50}\) and the maximum specific binding were compared for the mutant vs WT receptors by unpaired t-test. Significance was achieved at \( p < 0.05 \).

In the western blotting experiments, protein bands were analysed densitometrically using Multi Gauge software v2.2 (Fujifilm). To account for the difference in protein loading, the ratio of the band intensity detected with anti-myc antibody over the intensity of the corresponding GAPDH band from the same lane was first calculated. The band intensity ratios of the RAMP1 mutants were then normalised to that of WT RAMP1 as 100%. Data were analysed using one-way ANOVA (one way analysis of variance) followed by Dunnett’s test. Significance was achieved at \( p < 0.05 \).

The RAMP1 structure or RAMP3 model of the N-terminal domains were viewed and presented using PyMOL (http://www.pymol.org/).

Amino acid and nucleotide sequence alignments were performed using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).
Chapter 3

Pharmacological characterisation of RAMP1/3 chimaeras in AMY$_1$(a) receptors

3.1 Introduction

It is generally accepted that the RAMP N terminus is the key determinant of receptor pharmacology. This has been demonstrated in studies with either CLR or CT$_{(a)}$ employing RAMP1/2 chimaeras where the entire N terminus was exchanged between RAMPs 1 and 2 (Fraser et al., 1999; Zumpe et al., 2000). The N terminus of RAMP is most likely to directly contribute to peptide binding pocket together with the N terminus of the receptor, as cross-linking using $^{125}$I-AM and $^{125}$I-CGRP showed that the RAMPs located close to the peptide binding pocket in the CLR/RAMP complexes (Hilairet et al., 2001b). In the past decade, substantial mutagenesis data have been generated at the N terminus of RAMPs aiming to define regions or residues that are important for ligand to receptor interactions (Chapter 1 Table 1.2-1.4). SDM, chimaera and deletion mutations and photoaffinity cross-linking are the major approaches that have been used in these studies. However, the majority of these data were generated with CLR; little is known about the structure-function relationship of the RAMP residues in complex with CTR. In fact, W84 of RAMP1 is the only residue that has been implicated in AMY$_1$(a) pharmacology in a recent study (Gingell et al., 2010).

The RAMP to receptor association scenario is different between CLR and CTR. Unlike CLR, CTR expression at the cell surface is not dependent on RAMP association. However, the pharmacology of CTR is altered by the presence of RAMPs to produce the Amy receptor subtypes; AMY$_1$ (RAMP1/CTR), AMY$_2$ (RAMP2/CTR) and AMY$_3$ (RAMP3/CTR). Though the physiological significance of these three Amy receptor subtypes is not known, they do exhibit some differential pharmacology in transfected cell lines. An early study has shown that the generation of high affinity Amy receptor phenotypes from RAMP and CTR association is dependent on the CTR splice variant and cellular background (Tilakaratne et al., 2000). Further study has demonstrated that AMY$_1$(a), AMY$_3$(a) and CT$_{(a)}$ can be pharmacologically discriminated in Cos 7 cells by using a combination of agonists and antagonists (Hay et al., 2005). In particular, CGRP potency is ~10-fold higher at AMY$_1$(a) than at AMY$_3$(a) in Cos 7 cells (Hay et al., 2005).
Since AMY\textsubscript{1(a)} and AMY\textsubscript{3(a)} share CT\textsubscript{(a)}, thus the differential CGRP potencies exhibited by them are most likely to be determined by the differential sequences of RAMPs 1 and 3. In addition, given the long extracellular domains of the receptor components and large endogenous peptide ligands, it is very likely that multiple points of contact exist in the interactions between the calcitonin family of peptides and their receptors. Therefore, in the lack of experimentally supported information on the potential regions of the RAMP that may be involved in ligand to receptor interactions from the literature, and also to obtain an indication of the structure-function relationship of different regions throughout the N-terminal domain of RAMP1, a chimaera approach was used in this chapter.

The study performed in this chapter was carried out alongside another study where large chimaeras with one, two or three helices of the N terminus exchanged between RAMPs 1 and 3 were characterised with CT\textsubscript{(a)} (Qi et al., 2010). In general, incorporations of the foreign sequences mostly reduced CGRP (Tyr\textsuperscript{a}h\textalphaCGRP and h\betaCGRP) potencies. A pattern was observed. In RAMP3, CGRP potency was initially reduced by the introduction of RAMP1 helix 1 but was later gradually enhanced as more RAMP1 sequences was brought in. The data with these large chimaeras suggest some contribution of the RAMP sequence to the receptor pharmacology but did not provide a clear picture of which specific regions may be involved. In this chapter, a series of small chimaeras were made by replacing every four consecutive residues in an untagged version of human RAMP1 with the corresponding residues from human RAMP3 (Figure 3.1) to determine the regions in RAMP1 that are involved in conferring receptor specific pharmacology. Blocks of four residues were chosen to generate these small chimaeras as they broadly represent each turn in the helical structure of RAMPs 1 and 3.
Figure 3.1 Amino acid sequence alignment of human RAMPs 1 and 3. The predicted signal sequences are in blue and the TM domains are in red. 26 chimaeric RAMP1 with blocks of four residues replaced by the corresponding regions from RAMP3 were characterised in this chapter. The regions involved are indicated by solidus and the first residue in each block is numbered. For example, the first chimaera which had residues replaced at positions 18-21 in RAMP1 has a sequence of “…WLLLA CGGC FMTTA…” (residues 18-21 are underlined).

The small RAMP 1/3 chimaeras will be named with the numbering of the RAMP3 residues that have been introduced into RAMP1. For example, RAMP1 mutant with residues 18-21 replaced by the corresponding residues from RAMP3 will be referred as RAMP318-21 in the following sections.

These small chimaeras were characterised with human CT\(_{(a)}\) in this chapter. In terms of selecting agonists for characterising the effects of the regions replaced in these chimaeras, peptide potencies that have been previously determined at AMY\(_{1(a)}\) were compared to those at AMY\(_{3(a)}\) (Hay et al., 2005). All peptides with a moderate to high potency were more potent at AMY\(_{1(a)}\) than at AMY\(_{3(a)}\). The cognate peptide Amy was not a good choice as it showed little discrimination at these two Amy receptor subtypes. Therefore, Tyr\(^{\text{h}a}\)CGRP and h\(^{\beta}\)CGRP which showed the strongest discrimination between AMY\(_{1(a)}\) than AMY\(_{3(a)}\) (~30-fold difference in potency) were chosen to characterise these RAMP1/3 chimaeras with CT\(_{(a)}\) in this chapter. It was hypothesised that Tyr\(^{\text{h}a}\)CGRP and h\(^{\beta}\)CGRP potencies would be reduced at the AMY\(_{1(a)}\) receptors formed by the RAMP1/3 chimaeras and CT\(_{(a)}\) if the region replaced in RAMP1 was important for these peptide interactions with the receptor.
3.2 Results

3.2.1 Effects of RAMP1/3 chimaeras on receptor expression

3.2.1.1 Cell-surface expression

CT (a) has significant expression at the cell surface on its own (Hay et al., 2005) and thus measurement of its expression is not suitable for estimating expression of receptor complexes formed with RAMPs at the cell surface. On the other hand, it is technically difficult to measure the RAMP1/3 chimaera expression as these chimaeras are not tagged and no good specific native antibodies are available for RAMPs (DL Hay & RJ Bailey, unpublished data); besides, the antibody epitope might be lost in some chimaeras as a result of residues changes. Therefore, cell-surface expression of the RAMP1/3 chimaeras in complex with CLR was instead measured to provide an indication of the ability of the chimaeras to associate with a GPCR. As CLR can only be efficiently translocated to the cell surface in association with RAMP, measuring the HA-CLR expression at the cell surface was thus used as an indication of CLR and RAMP1 interactions. The chimaeric RAMP1 mutants were coexpressed with HA-CLR in Cos 7 cells and HA-CLR expression was measured in whole-cell ELISA.

Cell-surface expression of CLR in complex with the RAMP1/3 chimaeras is shown in Figure 3.2. As the chimaeras were made in RAMP1 with the RAMP3 sequences incorporated, therefore their expressions in complex with CLR were normalised to that of RAMP1, which was the control for the cell-surface expression analysis. Compared to CLR alone, the cotransfection of RAMP1 induced a significant increase in CLR cell-surface expression ($p < 0.001$ by one-way ANOVA followed by Dunnett’s test). RAMP3_{22-25}, RAMP3_{38-41}, RAMP3_{62-65}, RAMP3_{66-69}, RAMP3_{78-81}, RAMP3_{90-93} and RAMP3_{98-101} induced significant reductions in cell-surface expression of the RAMP1/CLR complex. Amongst them, chimaeras RAMP3_{22-25}, RAMP3_{38-41} and RAMP3_{62-65} failed to enhance CLR cell-surface expression, displaying expression levels which were comparable to HA-CLR alone expression at the cell surface.
Figure 3.2 ELISA data for RAMP1/3 chimaeras expressed with HA-CLR, measuring HA-CLR expression at the cell surface. Data are mean ± s.e.m. of three independent experiments, each performed with eight replicates. ** p < 0.01, *** p < 0.001 vs WT RAMP1/CLR by one-way ANOVA followed by Dunnett’s test.

3.2.1.2 Total expression of selected RAMP1/3 chimaeric constructs

To examine whether RAMP1/3 chimaeras had any significant impact on the intracellular protein synthesis, selected chimaeras which induced large reductions (≥ ~ 40% reduction compared to the WT) in CLR cell-surface expression were selected for analysis for their total cellular expression using western blotting. Four chimaeras RAMP322-25, RAMP338-41, RAMP362-65 and RAMP390-93 showed the largest reductions. RAMP322-25 resides within the predicted signal peptide of RAMP1, therefore the large reduction seen in cell-surface expression was most likely due to the distortion in receptor translocation. This is in contrast to RAMP318-21 which is also part of the signal sequence, but retained normal expression of the RAMP1/CLR complex at the cell surface. This suggests that these regions in the signal peptide may have different roles; it is possible that region 22-25, but not 18-21, provides some sequence recognition which is important for receptor translocation to the cell surface.

To determine the total expression of the other three chimaeras beyond the signal peptide, RAMP338-41, RAMP362-65 and RAMP390-93 were remade in a myc-tagged version of human
RAMP1 and their total cellular expression was determined in complex with HA-CLR (Figure 3.3). The western blotting data showed that all three chimaeras were produced at the expected size (~17kDa (Simms et al., 2008)) and the amount of protein made intracellularly was not significantly altered compared to WT RAMP1.

![Graph showing mycRAMP1 expression](image)

**Figure 3.3** Effects of three RAMP1/3 chimaeras on total cellular expression of mycRAMP1 in complex with HA-CLR. (a) Mean expression obtained from three independent quantitative western blotting experiments. Data were analysed by one-way ANOVA followed by Dunnett’s test; significance was achieved at $p < 0.05$. (b) Representative western blot.
3.2.2 Functional characterisation using cAMP assays

The untagged RAMP1/3 chimaeras mutants were individually cotransfected with HA-CT<sub>(a)</sub> into Cos 7 cells and Tyr<sup>a</sup>hαCGRP and hβCGRP responses at these mutant and WT AMY<sub>(a)</sub> receptors were determined in cAMP assays. Mean values of pEC<sub>50</sub> and E<sub>max</sub> from multiple experiments were calculated and representative response curves are presented. In addition, the N-terminal domains of the RAMP1 structure and RAMP3 model showing the residues that are involved to make these chimaeras are also shown. Of note, this does not include the two chimaeras from the signal peptide (RAMP3<sub>18-21</sub> and RAMP3<sub>22-25</sub>) and the last three chimaeras that are close to the TM domain (RAMP3<sub>110-113</sub>, RAMP3<sub>114-117</sub> and RAMP3<sub>118-121</sub>), as these regions were not included in the RAMP1 structure and RAMP3 model published by Kusano <i>et al.</i> (Kusano <i>et al.</i>, 2008) and Bailey <i>et al.</i> (Bailey <i>et al.</i>, 2010), respectively.

Tyr<sup>a</sup>hαCGRP and hβCGRP responses at WT AMY<sub>(a)</sub> and WT AMY<sub>(a)</sub> were first compared; they were ~7-fold and ~9-fold more potent at AMY<sub>(a)</sub> than at AMY<sub>(a)</sub>, respectively (Figure 3.4). The Tyr<sup>a</sup>hαCGRP responses were pEC<sub>50 ± s.e.m.</sub>, AMY<sub>(a)</sub> 8.61 ± 0.08 vs AMY<sub>(a)</sub> 7.77 ± 0.09 and E<sub>max ± s.e.m.</sub>, AMY<sub>(a)</sub> 68.7 ± 2.05 vs AMY<sub>(a)</sub> 77.6 ± 7.21 (n=3). The hβCGRP responses were pEC<sub>50 ± s.e.m.</sub>, AMY<sub>(a)</sub> 10.6 ± 0.28 vs AMY<sub>(a)</sub> 9.64 ± 0.15 and E<sub>max ± s.e.m.</sub>, AMY<sub>(a)</sub> 72.8 ± 6.57 vs AMY<sub>(a)</sub> 83.2 ± 7.49 (n=3).

![Figure 3.4](image-url) cAMP data for (a) Tyr<sup>a</sup>hαCGRP and (b) hβCGRP responses at WT AMY<sub>(a)</sub> and WT AMY<sub>(a)</sub>. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.1 RAMP3\textsubscript{18-21} AMY\textsubscript{1(a)}

There was no significant difference in peptide response between the mutant RAMP3\textsubscript{18-21} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)} receptor for either Tyr\textsuperscript{\textalpha}h\textalpha CGRP or h\textbeta CGRP (Figure 3.5). pEC\textsubscript{50} for Tyr\textsuperscript{\textalpha}h\textalpha CGRP at the mutant receptor was 8.44 ± 0.06 compared to 8.61 ± 0.08 at the WT receptor (pEC\textsubscript{50} ± s.e.m.; n=3). The E\textsubscript{max} values were 76.3 ± 3.41 for the mutant receptor and 68.7 ± 3.41 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m.). h\textbeta CGRP responses were: pEC\textsubscript{50} ± s.e.m., mutant 10.6 ± 0.20 vs WT 10.7 ± 0.24 and E\textsubscript{max} ± s.e.m., mutant 82.9 ± 5.21 vs WT 76.2 ± 5.21 (n=3).

**Figure 3.5** cAMP data for (a) Tyr\textsuperscript{\textalpha}h\textalpha CGRP and (b) h\textbeta CGRP responses at RAMP3\textsubscript{18-21} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.2 RAMP3$_{22-25}$ AMY$_{1(a)}$

Both Tyr°hαCGRP and hβCGRP potencies were significantly reduced at the mutant RAMP3$_{22-25}$ AMY$_{1(a)}$ receptor compared to the WT receptor (Figure 3.6). pEC$_{50}$ for Tyr°hαCGRP at the mutant receptor was 6.61 ± 0.09 compared to 8.67 ± 0.07 at the WT receptor (pEC$_{50}$ ± s.e.m., $p < 0.001$ by unpaired t-test, n=3), displaying a ~115-fold reduction. EC$_{max}$ was not significantly affected; 75.0 ± 2.37 for the mutant receptor and 70.3 ± 4.46 for the WT receptor, respectively (EC$_{max}$ ± s.e.m.). There was a ~288-fold reduction in hβCGRP potency (pEC$_{50}$ ± s.e.m., mutant 8.09 ± 0.19 vs WT 10.6 ± 0.22, $p < 0.001$ by unpaired t-test, n=4) whereas the maximum response was not significantly altered (EC$_{max}$ ± s.e.m., mutant 76.5 ± 6.64 vs WT 72.6 ± 6.57).

**Figure 3.6** cAMP data for (a) Tyr°hαCGRP and (b) hβCGRP responses at RAMP3$_{22-25}$ AMY$_{1(a)}$ and WT AMY$_{1(a)}$ receptors. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.3 RAMP326-29 AMY1(a)

There was no significant difference in peptide response between the mutant RAMP326-29 AMY1(a) and WT AMY1(a) receptor for either Tyr°hαCGRP or hβCGRP (Figure 3.7). pEC$_{50}$ for Tyr°hαCGRP at the mutant receptor was 7.75 ± 0.49 compared to 8.72 ± 0.07 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=4). The E$_{\text{max}}$ values were 65.0 ± 7.61 for the mutant receptor and 65.0 ± 4.46 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). hβCGRP responses were: pEC$_{50}$ ± s.e.m., mutant 9.61 ± 0.26 vs WT 10.5 ± 0.28 and E$_{\text{max}}$ ± s.e.m., mutant 71.9 ± 10.7 vs WT 70.2 ± 6.01 (n=4).

Figure 3.7 (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr°hαCGRP and (c) hβCGRP responses at RAMP326-29 AMY1(a) and WT AMY1(a) receptors. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.4 RAMP3\textsubscript{30-33} AMY\textsubscript{1(a)}

Both Tyr\textsuperscript{\#}haCGRP and h\textbeta{CGRP} potencies were significantly reduced at the mutant RAMP3\textsubscript{30-33} AMY\textsubscript{1(a)} receptor compared to the WT receptor (Figure 3.8). pEC\textsubscript{50} for Tyr\textsuperscript{\#}haCGRP at the mutant receptor was 6.34 ± 0.12 compared to 8.67 ± 0.11 at the WT receptor (pEC\textsubscript{50} ± s.e.m., p < 0.001 by unpaired t-test, n=3), displaying a ~214-fold reduction in potency. E\textsubscript{max} was not significantly affected; 56.2 ± 17.2 for the mutant receptor and 52.3 ± 1.60 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m.). There was a ~138-fold reduction in h\textbeta{CGRP} potency (pEC\textsubscript{50} ± s.e.m., mutant 7.81 ± 0.04 vs WT 9.95 ± 0.42, p < 0.01 by unpaired t-test, n=3) whereas the maximum response was not significantly altered (E\textsubscript{max} ± s.e.m., mutant 60.5 ± 8.75 vs WT 54.7 ± 7.10).

![Figure 3.8](image)

**Figure 3.8** (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano *et al*., 2008) and RAMP3 model (cyan, Bailey *et al*., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr\textsuperscript{\#}haCGRP and (c) h\textbeta{CGRP} responses at RAMP3\textsubscript{30-33} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.5 RAMP3_{34-37} AMY_{1(a)}

Both Tyr^αhαCGRP and hβCGRP potencies were significantly reduced at the mutant RAMP3_{34-37} AMY_{1(a)} receptor compared to the WT receptor (Figure 3.9). pEC_{50} for Tyr^αhαCGRP at the mutant receptor was 6.61 ± 0.09 compared to 8.78 ± 0.11 at the WT receptor (pEC_{50} ± s.e.m., p < 0.001 by unpaired t-test, n=4), displaying a ~148-fold reduction in potency. E_{max} was not significantly affected; 81.9 ± 6.90 for the mutant receptor and 74.6 ± 1.57 for the WT receptor, respectively (E_{max} ± s.e.m.). There was a ~513-fold reduction in hβCGRP potency (pEC_{50} ± s.e.m., mutant 8.00 ± 0.20 vs WT 10.7 ± 0.19, p < 0.001 by unpaired t-test, n=4) whereas the maximum response was not significantly altered (E_{max} ± s.e.m., mutant 76.9 ± 6.60 vs WT 73.1 ± 4.90).

Figure 3.9 (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr^αhαCGRP and (c) hβCGRP responses at RAMP3_{34-37} AMY_{1(a)} and WT AMY_{1(a)}. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.6 RAMP3\textsubscript{38-41} AMY\textsubscript{1(a)}

Both Tyr\textsuperscript{o}h\alpha CGRP and h\beta CGRP potencies were significantly reduced at the mutant RAMP3\textsubscript{38-41} AMY\textsubscript{1(a)} receptor compared to the WT receptor (Figure 3.10). pEC\textsubscript{50} for Tyr\textsuperscript{o}h\alpha CGRP at the mutant receptor was 5.97 ± 0.24 compared to 8.60 ± 0.06 at the WT receptor (pEC\textsubscript{50} ± s.e.m., p < 0.001 by unpaired t-test, n=4), displaying a ~427-fold reduction in potency. $E_{\text{max}}$ was not significantly affected; 75.5 ± 6.85 for the mutant receptor and 53.1 ± 10.2 for the WT receptor, respectively ($E_{\text{max}}$ ± s.e.m.). There was a ~295-fold reduction in h\beta CGRP potency (pEC\textsubscript{50} ± s.e.m., mutant 7.82 ± 0.15 vs WT 10.3 ± 0.28, p < 0.001 by unpaired t-test, n=5) whereas the maximum response was not significantly altered ($E_{\text{max}}$ ± s.e.m., mutant 57.6 ± 10.2 vs WT 63.5 ± 5.80).

**Figure 3.10** (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr\textsuperscript{o}h\alpha CGRP and (c) h\beta CGRP responses at RAMP3\textsubscript{38-41} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of four-five independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.7 RAMP342-45 AMY1(a)

There was no significant difference in peptide response between the mutant RAMP342-45 AMY1(a) and the WT receptor for either Tyr°hαCGRP or hβCGRP (Figure 3.11). pEC$_{50}$ for Tyr°hαCGRP at the mutant receptor was $8.17 \pm 0.36$ compared to $8.78 \pm 0.13$ at the WT receptor (pEC$_{50}$ ± s.e.m.; n=4). The E$_{\text{max}}$ values were $66.5 \pm 11.3$ for the mutant receptor and $68.1 \pm 6.28$ for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). hβCGRP responses were: pEC$_{50}$ ± s.e.m., mutant $9.94 \pm 0.38$ vs WT $10.5 \pm 0.46$ and E$_{\text{max}}$ ± s.e.m., mutant $69.4 \pm 10.5$ vs WT $66.8 \pm 9.27$ (n=4).

![Figure 3.11](image)

**Figure 3.11** (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr°hαCGRP and (c) hβCGRP responses at RAMP342-45 AMY1(a) and WT AMY1(a). The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.8 RAMP3<sub>46-49</sub> AMY<sub>1(a)</sub>

Both Tyr°hαCGRP and hβCGRP potencies were significantly reduced at the mutant RAMP3<sub>46-49</sub> AMY<sub>1(a)</sub> receptor compared to the WT receptor (Figure 3.12). pEC<sub>50</sub> for Tyr°hαCGRP at the mutant receptor was 7.01 ± 0.19 compared to 8.56 ± 0.06 at the WT receptor (pEC<sub>50</sub> ± s.e.m., p < 0.001 by unpaired t-test, n=4), displaying a ~35-fold reduction in potency. E<sub>max</sub> was not significantly affected; 49.3 ± 10.2 for the mutant receptor and 56.4 ± 11.4 for the WT receptor, respectively (E<sub>max</sub> ± s.e.m.). There was a ~76-fold reduction in hβCGRP potency (pEC<sub>50</sub> ± s.e.m., mutant 8.47 ± 0.23 vs WT 10.4 ± 0.34, p < 0.01 by unpaired t-test, n=4) whereas the maximum response was not significantly altered (E<sub>max</sub> ± s.e.m., mutant 61.9 ± 11.41 vs WT 65.9 ± 5.40, n=4).

**Figure 3.12** (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr°hαCGRP and (c) hβCGRP responses at RAMP3<sub>46-49</sub> AMY<sub>1(a)</sub> and WT AMY<sub>1(a)</sub>. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.9 RAMP3\textsubscript{50-53} AMY\textsubscript{1(a)}

Both Tyr\textsuperscript{\theta}h\alpha CGRP and h\beta CGRP potencies were significantly reduced at the mutant RAMP3\textsubscript{50-53} AMY\textsubscript{1(a)} receptor compared to the WT receptor (Figure 3.13). pEC\textsubscript{50} for Tyr\textsuperscript{\theta}h\alpha CGRP at the mutant receptor was 8.16 ± 0.10 compared to 9.23 ± 0.04 at the WT receptor (pEC\textsubscript{50} ± s.e.m., p < 0.001 by unpaired t-test, n=3), displaying a ~12-fold reduction in potency. E\textsubscript{max} was also significantly reduced; 67.7 ± 1.40 for the mutant receptor and 79.8 ± 2.07 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m., p < 0.01 by unpaired t-test, n=3). There was a ~6-fold reduction in h\beta CGRP potency (pEC\textsubscript{50} ± s.e.m., mutant 9.78 ± 0.21 vs WT 10.5 ± 0.07, p < 0.05 by unpaired t-test, n=4) whereas the maximum response was not significantly altered (E\textsubscript{max} ± s.e.m., mutant 81.2 ± 3.43 vs WT 77.9 ± 0.91).

Figure 3.13 (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano \textit{et al.}, 2008) and RAMP3 model (cyan, Bailey \textit{et al.}, 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr\textsuperscript{\theta}h\alpha CGRP and (c) h\beta CGRP responses at RAMP3\textsubscript{50-53} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of three-four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.10 RAMP3<sub>54-57</sub> AMY<sub>1(a)</sub>

Both Tyr°hαCGRP and hβCGRP potencies were significantly reduced at the mutant RAMP3<sub>54-57</sub> AMY<sub>1(a)</sub> receptor compared to the WT receptor (Figure 3.14). pEC<sub>50</sub> for Tyr°hαCGRP at the mutant receptor was 7.75 ± 0.10 compared to 9.23 ± 0.04 at the WT receptor (pEC<sub>50</sub> ± s.e.m., p < 0.001 by unpaired t-test, n=3), displaying a ~30-fold reduction in potency. E<sub>max</sub> was also significantly reduced; 56.5 ± 1.28 for the mutant receptor and 79.8 ± 2.07 for the WT receptor, respectively (E<sub>max</sub> ± s.e.m., p < 0.001 by unpaired t-test, n=3). There was a ~9-fold reduction in hβCGRP potency (pEC<sub>50</sub> ± s.e.m., mutant 9.60 ± 0.07 vs WT 10.6 ± 0.09, p < 0.01 by unpaired t-test, n=3) whereas the maximum response was not significantly altered (E<sub>max</sub> ± s.e.m., mutant 78.5 ± 6.65 vs WT 77.5 ± 1.14).

Figure 3.14 (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr°hαCGRP and (c) hβCGRP responses at RAMP3<sub>54-57</sub> AMY<sub>1(a)</sub> and WT AMY<sub>1(a)</sub>. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.11 RAMP3<sub>58-61</sub> AMY<sub>1(a)</sub>

Both Tyr<sup>9</sup>hαCGRP and hβCGRP potencies were significantly reduced at the mutant RAMP3<sub>58-61</sub> AMY<sub>1(a)</sub> receptor compared to the WT receptor (Figure 3.15). pEC<sub>50</sub> for Tyr<sup>9</sup>hαCGRP at the mutant receptor was 8.50 ± 0.13 compared to 9.30 ± 0.04 for the WT receptor (pEC<sub>50</sub> ± s.e.m., p < 0.01 by unpaired t-test, n=3), displaying a ~6-fold reduction in potency. E<sub>max</sub> was also significantly reduced; 67.8 ± 3.65 for the mutant receptor and 79.8 ± 1.87 for the WT receptor, respectively (E<sub>max</sub> ± s.e.m, p < 0.05 by unpaired t-test, n=4). There was a ~3-fold reduction in hβCGRP potency (pEC<sub>50</sub> ± s.e.m., mutant 9.94± 0.15 vs WT 10.5 ± 0.03, p < 0.05 by unpaired t-test, n=3) whereas the maximum response was not significantly altered (E<sub>max</sub> ± s.e.m., mutant 87.8 ± 6.21 vs WT 83.6 ± 6.49).

![Figure 3.15](image)

**(a)** The N-terminal domain of the RAMP1 structure (dark blue, Kusano *et al.*, 2008) and RAMP3 model (cyan, Bailey *et al.*, 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr<sup>9</sup>hαCGRP and (c) hβCGRP responses at RAMP3<sub>58-61</sub> AMY<sub>1(a)</sub> and WT AMY<sub>1(a)</sub>. The graphs are representative of three-four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.12 RAMP3<sub>62-65</sub> AMY<sub>1(a)</sub>

Both Tyr<sup>°</sup>hαCGRP and hβCGRP potencies were significantly reduced at the mutant RAMP3<sub>62-65</sub> AMY<sub>1(a)</sub> receptor compared to the WT receptor (Figure 3.16). pEC<sub>50</sub> for Tyr<sup>°</sup>hαCGRP at the mutant receptor was 6.82 ± 0.15 compared to 9.12 ± 0.06 for the WT receptor (pEC<sub>50</sub> ± s.e.m., p < 0.001 by unpaired t-test, n=3), displaying a ~200-fold reduction in potency. E<sub>max</sub> was not significantly affected; 80.7 ± 6.39 for the mutant receptor and 79.3 ± 2.57 for the WT receptor, respectively (E<sub>max</sub> ± s.e.m.). There was a ~107-fold reduction in hβCGRP potency (pEC<sub>50</sub> ± s.e.m., mutant 8.42 ± 0.11 vs WT 10.5 ± 0.03, p < 0.001 by unpaired t-test, n=3) whereas the maximum response was not significantly altered (E<sub>max</sub> ± s.e.m., mutant 87.5 ± 2.75 vs WT 83.6 ± 6.49).

Figure 3.16 (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr<sup>°</sup>hαCGRP and (c) hβCGRP responses at RAMP3<sub>62-65</sub> AMY<sub>1(a)</sub> and WT AMY<sub>1(a)</sub>. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.13 RAMP3\textsubscript{66-69} AMY\textsubscript{1(a)}

Both Tyr\textsuperscript{o}h\textalpha{}CGRP and h\textbeta{}CGRP potencies were significantly reduced at the mutant RAMP3\textsubscript{66-69} AMY\textsubscript{1(a)} receptor compared to the WT receptor (Figure 3.17). pEC\textsubscript{50} for Tyr\textsuperscript{o}h\textalpha{}CGRP response at the mutant receptor was 7.40 ± 0.21 compared to 9.26 ± 0.01 for the WT receptor (pEC\textsubscript{50} ± s.e.m., p < 0.001 by unpaired t-test, n=4), displaying a ~72-fold reduction in potency. \(E_{\text{max}}\) was also significantly reduced: 66.6 ± 2.59 for the mutant receptor and 79.1 ± 2.56 for the WT receptor, respectively (\(E_{\text{max}}\) ± s.e.m., p < 0.05 by unpaired t-test, n=4). There was a ~36-fold reduction in h\textbeta{}CGRP potency (pEC\textsubscript{50} ± s.e.m., mutant 9.01 ± 0.19 vs WT 10.6 ± 0.09, p < 0.001 by unpaired t-test, n=4) whereas the maximum response was not significantly altered (\(E_{\text{max}}\) ± s.e.m., mutant 82.8 ± 3.61 vs WT 79.7 ± 2.01).

\textbf{Figure 3.17} (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano \textit{et al.}, 2008) and RAMP3 model (cyan, Bailey \textit{et al.}, 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr\textsuperscript{o}h\textalpha{}CGRP and (c) h\textbeta{}CGRP responses at RAMP3\textsubscript{66-69} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.14 RAMP3\textsubscript{70-73} AMY\textsubscript{1(a)}

There was no significant difference in peptide response between the mutant RAMP3\textsubscript{70-73} AMY\textsubscript{1(a)} and WT receptors for either Tyr\textsuperscript{º}h\textalpha{}CGRP or h\textbeta{}CGRP (Figure 3.18). pEC\textsubscript{50} for Tyr\textsuperscript{º}h\textalpha{}CGRP at the mutant receptor was 9.48 ± 0.16 compared to 9.27 ± 0.01 at the WT receptor (pEC\textsubscript{50} ± s.e.m.; n=3). The E\textsubscript{max} values were 77.1 ± 4.09 for the mutant receptor and 78.4 ± 3.49 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m.). h\textbeta{}CGRP responses were: pEC\textsubscript{50} ± s.e.m., mutant 10.9 ± 0.02 vs WT 10.6 ± 0.13 and E\textsubscript{max} ± s.e.m., mutant 83.7 ± 5.59 vs WT 79.8 ± 2.84 (n=3).

**Figure 3.18** (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr\textsuperscript{º}h\textalpha{}CGRP and (c) h\textbeta{}CGRP responses at RAMP3\textsubscript{70-73} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.15 RAMP3\textsubscript{74-77} AMY\textsubscript{1(a)}

Tyr\(^\circ\)h\(\alpha\)CGRP potencies was significantly reduced at the mutant RAMP3\textsubscript{74-77} AMY\textsubscript{1(a)} receptor compared to the WT receptor (Figure 3.19b). pEC\textsubscript{50} for Tyr\(^\circ\)h\(\alpha\)CGRP at the mutant receptor was 8.49 ± 0.24 compared to 9.27 ± 0.01 at the WT receptor (pEC\textsubscript{50} ± s.e.m., \(p < 0.05\) by unpaired t-test, \(n=3\)), displaying ~6-fold reduction in potency. E\textsubscript{max} was also significantly reduced; 66.9 ± 4.43 for the mutant receptor and 82.1 ± 0.48 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m., \(p < 0.05\) by unpaired t-test, \(n=3\)). On the other hand, h\(\beta\)CGRP response was not affected by the mutation (Figure 3.19c): pEC\textsubscript{50} ± s.e.m., mutant 10.1 ± 0.19 vs WT 10.4 ± 0.03, \(n=3\); E\textsubscript{max} ± s.e.m., mutant 80.2 ± 3.86 vs WT 76.6 ± 1.29).

**Figure 3.19** (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr\(^\circ\)h\(\alpha\)CGRP and (c) h\(\beta\)CGRP responses at RAMP3\textsubscript{74-77} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.16 RAMP3\textsubscript{78-81} AMY\textsubscript{1(a)}

Both Tyr\textsuperscript{0}h\textalpha{}CGRP and h\textbeta{}CGRP potencies were significantly reduced at the mutant RAMP3\textsubscript{78-81} AMY\textsubscript{1(a)} receptor compared to the WT receptor (Figure 3.20). pEC\textsubscript{50} for Tyr\textsuperscript{0}h\textalpha{}CGRP at the mutant receptor was 8.08 ± 0.04 compared to 9.27 ± 0.01 at the WT receptor (pEC\textsubscript{50} ± s.e.m., p < 0.001 by unpaired t-test, n=3), displaying a ~15-fold reduction in potency. E\textsubscript{max} was also significantly reduced as 63.2 ± 1.79 for the mutant receptor and 82.1 ± 0.48 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m., p < 0.001 by unpaired t-test, n=3). There was a ~7-fold reduction in h\textbeta{}CGRP potency (pEC\textsubscript{50} ± s.e.m., mutant 9.58 ± 0.16 vs WT 10.44 ± 0.03, p < 0.01 by unpaired t-test, n=3) whereas the maximum response was not significantly altered (E\textsubscript{max} ± s.e.m., mutant 78.6 ± 4.17 vs WT 76.6 ± 1.29) (Figure 3.20c).

\textbf{Figure 3.20} (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano \textit{et al.}, 2008) and RAMP3 model (cyan, Bailey \textit{et al.}, 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr\textsuperscript{0}h\textalpha{}CGRP and (c) h\textbeta{}CGRP responses at RAMP3\textsubscript{78-81} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.17 RAMP\textsubscript{82-85} AMY\textsubscript{1(a)}

Tyr\textsuperscript{\alpha}h\alphaCGRP potency was significantly reduced at the mutant RAMP\textsubscript{82-85} AMY\textsubscript{1(a)} receptor compared to the WT receptor (Figure 3.21b). pEC\textsubscript{50} for Tyr\textsuperscript{\alpha}h\alphaCGRP at the mutant receptor was 8.66 ± 0.24 compared to 9.46 ± 0.11 at the WT receptor (pEC\textsubscript{50} ± s.e.m., p < 0.05 by unpaired t-test, n=4), displaying a ~6-fold reduction in potency. E\textsubscript{max} was not significantly affected; 72.4 ± 3.82 for the mutant receptor and 75.4 ± 3.99 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m.). On the other hand, there was some reduction in h\betaCGRP potency but it was not statistically significant (Figure 3.21c): pEC\textsubscript{50} ± s.e.m., mutant 10.1 ± 0.53 vs WT 11.4 ± 0.14, n=3); E\textsubscript{max} ± s.e.m., mutant 82.2 ± 1.31 vs WT 72.3 ± 5.61, n=3).

![Figure 3.21](image)

**Figure 3.21** (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr\textsuperscript{\alpha}h\alphaCGRP and (c) h\betaCGRP responses at RAMP\textsubscript{82-85} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of three-four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.18 RAMP3<sub>86-89</sub> AMY<sub>1(a)</sub>

Both Tyr<sup>°</sup>hαCGRP and hβCGRP potencies were significantly reduced at the mutant RAMP3<sub>86-89</sub> AMY<sub>1(a)</sub> receptor compared to the WT receptor (Figure 3.22). pEC<sub>50</sub> for Tyr<sup>°</sup>hαCGRP at the mutant receptor was 7.28 ± 0.03 compared to 9.60 ± 0.03 at the WT receptor (pEC<sub>50</sub> ± s.e.m., p < 0.001 by unpaired t-test, n=3), displaying a ~209-fold reduction in potency. E<sub>max</sub> was not significantly affected; 74.7 ± 7.74 for the mutant receptor and 77.5 ± 3.75 for the WT receptor, respectively (E<sub>max</sub> ± s.e.m.). There was a ~347-fold reduction in hβCGRP potency (pEC<sub>50</sub> ± s.e.m., mutant 8.66 ± 0.11 vs WT 11.2 ± 0.20, p < 0.001 by unpaired t-test, n=4) and the maximum response was significantly enhanced (E<sub>max</sub> ± s.e.m., mutant 87.6 ± 3.08 vs WT 72.2 ± 3.97, p < 0.05 by unpaired t-test).

**Figure 3.22** (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr<sup>°</sup>hαCGRP and (c) hβCGRP responses at RAMP3<sub>86-89</sub> AMY<sub>1(a)</sub> and WT AMY<sub>1(a)</sub>. The graphs are representative of three-four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.19 RAMP3\textsubscript{90-93} AMY\textsubscript{1(a)}

Both Tyr\textsubscript{hα}CGRP and hβCGRP potencies were significantly reduced at the mutant RAMP3\textsubscript{90-93} AMY\textsubscript{1(a)} receptor compared to the WT receptor (Figure 3.23). pEC\textsubscript{50} for Tyr\textsubscript{hα}CGRP at the mutant receptor was 6.98 ± 0.05 compared to 9.55 ± 0.08 at the WT receptor (pEC\textsubscript{50} ± s.e.m., $p < 0.001$ by unpaired t-test, n=3), displaying a ~372-fold reduction in potency. $E_{\text{max}}$ was not significantly affected; 81.7 ± 9.39 for the mutant receptor and 79.1 ± 2.42 for the WT receptor, respectively ($E_{\text{max}}$ ± s.e.m.). There was a ~1175-fold reduction in hβCGRP potency (pEC\textsubscript{50} ± s.e.m., mutant 8.30 ± 0.11 vs WT 11.4 ± 0.14, $p < 0.001$ by unpaired t-test, n=3) whereas the maximum response was not significantly affected ($E_{\text{max}}$ ± s.e.m., mutant 91.3 ± 7.40 vs WT 72.3 ± 5.61).

Figure 3.23 (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr\textsubscript{hα}CGRP and (c) hβCGRP responses at RAMP3\textsubscript{90-93} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.20 RAMP3\textsuperscript{94-97} AMY\textsubscript{1(a)}

Both Tyr\textsuperscript{a}hαCGRP and hβCGRP potencies were significantly reduced at the mutant RAMP3\textsuperscript{94-97} AMY\textsubscript{1(a)} receptor compared to the WT receptor (Figure 3.24). $p_{EC_{50}}$ for Tyr\textsuperscript{a}hαCGRP at the mutant receptor was 8.41 ± 0.26 compared to 9.46 ± 0.11 at the WT receptor ($p_{EC_{50}}$ ± s.e.m., $p < 0.01$ by unpaired t-test, n=4), displaying a ~11-fold reduction in potency. $E_{max}$ was not significantly affected; 73.4 ± 4.71 for the mutant receptor and 75.4 ± 3.99 for the WT receptor, respectively ($E_{max}$ ± s.e.m.). There was a ~9-fold reduction in hβCGRP potency ($p_{EC_{50}}$ ± s.e.m., mutant 10.5 ± 0.07 vs WT 11.4 ± 0.14, $p < 0.01$ by unpaired t-test, n=3) whereas the maximum response was not significantly affected ($E_{max}$ ± s.e.m., mutant 66.0 ± 5.11 vs WT 72.3 ± 5.61).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure3.24.png}
\caption{(a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr\textsuperscript{a}hαCGRP and (c) hβCGRP responses at RAMP3\textsuperscript{94-97} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of three-four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.}
\end{figure}
3.2.2.21 RAMP3<sub>98-101</sub> AMY<sub>1(a)</sub>

Both Tyr<sup>9</sup>hαCGRP and hβCGRP potencies were significantly reduced at the mutant RAMP3<sub>98-101</sub> AMY<sub>1(a)</sub> receptor compared to the WT receptor (Figure 3.25). pEC<sub>50</sub> for Tyr<sup>9</sup>hαCGRP at the mutant receptor was 8.44 ± 0.23 compared to 9.55 ± 0.08 at the WT receptor (pEC<sub>50</sub> ± s.e.m., p < 0.05 by unpaired t-test, n=3), displaying a ~13-fold reduction in potency. E<sub>max</sub> was not significantly affected; 74.2 ± 4.51 for the mutant receptor and 79.1 ± 2.42 for the WT receptor, respectively (E<sub>max</sub> ± s.e.m.). There was a ~10-fold reduction in hβCGRP potency (pEC<sub>50</sub> ± s.e.m., mutant 10.4 ± 0.06 vs WT 11.4 ± 0.14, p < 0.01 by unpaired t-test, n=3) whereas the maximum response was not significantly affected (E<sub>max</sub> ± s.e.m., mutant 74.4 ± 1.19 vs WT 72.3 ± 5.61).

Figure 3.25 (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr<sup>9</sup>hαCGRP and (c) hβCGRP responses at RAMP3<sub>98-101</sub> AMY<sub>1(a)</sub> and WT AMY<sub>1(a)</sub>. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.22 RAMP3\textsubscript{102-105} AMY\textsubscript{1(a)}

Both Tyr\textsuperscript{o}h\textalpha{}CGRP and hßCGRP potencies were significantly reduced at the mutant RAMP3\textsubscript{102-105} AMY\textsubscript{1(a)} receptor compared to the WT receptor (Figure 3.26). The pEC\textsubscript{50} for Tyr\textsuperscript{o}h\textalpha{}CGRP at the mutant receptor was 8.99 ± 0.18 compared to 9.55 ± 0.08 for the WT receptor (pEC\textsubscript{50} ± s.e.m., p < 0.05 by unpaired t-test, n=3), displaying a \sim{}4-fold reduction in potency. E\textsubscript{max} was not significantly affected; 73.2 ± 7.99 for the mutant receptor and 79.1 ± 2.42 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m.). There was a \sim{}6-fold reduction in hßCGRP potency (pEC\textsubscript{50} ± s.e.m., mutant 10.6 ± 0.03 vs WT 11.4 ± 0.14, p < 0.01 by unpaired t-test, n=3) whereas the maximum response was not significantly affected (E\textsubscript{max} ± s.e.m., mutant 79.4 ± 5.82 vs WT 72.3 ± 5.61).

![Figure 3.26](image-url)

Figure 3.26 (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano et al., 2008) and RAMP3 model (cyan, Bailey et al., 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr\textsuperscript{o}h\textalpha{}CGRP and (c) hßCGRP responses at RAMP3\textsubscript{102-105} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.23 RAMP3_{106-109} AMY_{1(a)}

There was no significant difference in peptide response between the mutant RAMP3_{106-109} AMY_{1(a)} and WT receptor for either Tyr^{\alpha}h\alphaCGRP or h\betaCGRP (Figure 3.27). pEC_{50} for Tyr^{\alpha}h\alphaCGRP at the mutant receptor was 8.60 ± 0.35 compared to 9.37 ± 0.13 at the WT receptor (pEC_{50} ± s.e.m.; n=4). The E_{\text{max}} values were 83.4 ± 8.08 for the mutant receptor and 82.9 ± 4.43 for the WT receptor, respectively (E_{\text{max}} ± s.e.m.). h\betaCGRP responses were: pEC_{50} ± s.e.m., mutant 10.6 ± 0.24 vs WT 11.1 ± 0.17 and E_{\text{max}} ± s.e.m., mutant 64.2 ± 6.06 vs WT 69.2 ± 3.13 (n=3).

![Image](image-url)

**Figure 3.27** (a) The N-terminal domain of the RAMP1 structure (dark blue, Kusano *et al.*, 2008) and RAMP3 model (cyan, Bailey *et al.*, 2010) showing the residues (red) that have been replaced in RAMP1 with the corresponding region in RAMP3. (b) cAMP data for Tyr^{\alpha}h\alphaCGRP and (c) h\betaCGRP responses at RAMP3_{106-109} AMY_{1(a)} and WT AMY_{1(a)}. The graphs are representative of three-four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
Chapter 3

3.2.2.24 RAMP3\textsubscript{110-113} AMY\textsubscript{1(a)}

There was no significant difference in peptide response between the mutant RAMP3\textsubscript{110-113} AMY\textsubscript{1(a)} and WT receptors for either Tyr\textsuperscript{a}h\alpha CGRP or h\beta CGRP (Figure 3.28). pEC\textsubscript{50} for Tyr\textsuperscript{a}h\alpha CGRP at the mutant receptor was $9.18 \pm 0.31$ compared to $9.29 \pm 0.14$ at the WT receptor (pEC\textsubscript{50} ± s.e.m.; n=3). The E\textsubscript{max} values were $80.9 \pm 5.47$ for the mutant receptor and $84.5 \pm 5.84$ for the WT receptor, respectively (E\textsubscript{max} ± s.e.m.). h\beta CGRP responses were: pEC\textsubscript{50} ± s.e.m., mutant $10.7 \pm 0.19$ vs WT $10.9 \pm 0.17$ and E\textsubscript{max} ± s.e.m., mutant $80.2 \pm 4.47$ vs WT $69.3 \pm 3.18$ (n=3).

![Graph](image)

**Figure 3.28** cAMP data for (a) Tyr\textsuperscript{a}h\alpha CGRP and (b) h\beta CGRP responses at RAMP3\textsubscript{110-113} AMY\textsubscript{1(a)} and WT AMY\textsubscript{1(a)}. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.25 RAMP3<sub>114-117</sub> AMY<sub>1(a)</sub>

There was a small but significant reduction in the maximum response of Tyr°hαCGRP at the mutant RAMP3<sub>114-117</sub> AMY<sub>1(a)</sub> compared to the WT receptor (E<sub>max</sub> ± s.e.m., mutant 70.3 ± 1.97 vs WT 78.7 ± 2.09, p < 0.05 by unpaired t-test, n=3). On the other hand, Tyr°hαCGRP potency was not affected at the mutant receptor: pEC<sub>50</sub> ± s.e.m., mutant 9.21 ± 0.27 vs WT 9.49 ± 0.07 (Figure 3.29a). There was no significant difference between and mutant and WT receptor for hβCGRP responses: pEC<sub>50</sub> ± s.e.m., mutant 10.7 ± 0.19 vs WT 10.9 ± 0.17; E<sub>max</sub> ± s.e.m., mutant 80.2 ± 4.47 vs WT 69.3 ± 3.18 (n=3) (Figure 3.29b).

**Figure 3.29** cAMP data for (a) Tyr°hαCGRP and (b) hβCGRP responses at RAMP3<sub>114-117</sub> AMY<sub>1(a)</sub> and WT AMY<sub>1(a)</sub>. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.2.26 RAMP$^{118-121}$ AMY$_{1(a)}$

There was a small but significant enhancement for Tyr$^{\alpha}$hαCGRP potency at the mutant RAMP$^{118-121}$ AMY$_{1(a)}$ receptor compared to the WT receptor (Figure 3.30a). pEC$_{50}$ for Tyr$^{\alpha}$hαCGRP at the mutant receptor was 9.96 ± 0.12 compared to 9.51 ± 0.06 at the WT receptor (pEC$_{50}$ ± s.e.m., $p < 0.05$ by unpaired t-test, n=3), displaying a ~3-fold enhancement in potency. $E_{\text{max}}$ was not significantly affected; 80.9 ± 2.69 for the mutant receptor and 81.1 ± 1.51 for the WT receptor, respectively ($E_{\text{max}}$ ± s.e.m.). On the other hand, hβCGRP response was not significantly altered: pEC$_{50}$ ± s.e.m., mutant 11.1 ± 0.10 vs WT 10.9 ± 0.19 and $E_{\text{max}}$ ± s.e.m., mutant 86.6 ± 9.55 vs WT 72.5 ± 0.46 (n=3) (Figure 3.30b).

**Figure 3.30** cAMP data for (a) Tyr$^{\alpha}$hαCGRP and (b) hβCGRP responses at RAMP$^{118-121}$ AMY$_{1(a)}$ and WT AMY$_{1(a)}$. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
3.2.3 rAmy responses at selected chimaeric AMY\textsubscript{1(a)} mutants

As the majority of RAMP1/3 chimaeras induced significant changes to both forms of CGRP responses, it becomes a question whether these changes seen in peptide responses were genuine changes resulting from alterations in the binding pocket by the mutations, or simply caused by altered receptor expression at the cell surface. However, as explained earlier, cell-surface expression of these mutant AMY\textsubscript{1(a)} receptors could not be directly measured, and the total and cell-surface expression data generated with the chimaeras in complex with CLR indicated that most chimaeras should be synthesised at normal levels in the cells and they retain the ability to associate with CLR. Therefore, rAmy response was determined for selected chimaeras. rAmy was equally potent at AMY\textsubscript{1(a)} and AMY\textsubscript{3(a)} (pEC\textsubscript{50} ± s.e.m., AMY\textsubscript{1(a)} 10.3 ± 0.12 vs AMY\textsubscript{3(a)} 10.5 ± 0.19 and E\textsubscript{max} ± s.e.m., AMY\textsubscript{1(a)} 79.4 ± 2.85 vs AMY\textsubscript{3(a)} 85.6 ± 3.85, n=3). Thus, it was hypothesised that the introduction of RAMP3 residues into RAMP1 would not alter rAmy response at the mutant AMY\textsubscript{1(a)} receptors, unless the chimaeras have introduced structural distortions to the protein and led to altered receptor expression at the cell surface. In this context, rAmy response can be used to provide an indication of a gross distortion of AMY\textsubscript{1(a)} receptor formation as any change seen with rAmy response would most likely be resulted from an altered receptor expression at the cell surface based on the hypothesis.

rAmy responses were determined at six RAMP1/3 chimaeras with CT\textsubscript{(a)}, they are chimaeras that showed no change in CGRP potency (RAMP\textsubscript{342-45} and RAMP\textsubscript{370-73}), chimaeras that showed modest (~10-fold) reductions in CGRP potency (RAMP\textsubscript{350-53}, RAMP\textsubscript{358-61} and RAMP\textsubscript{394-97}), and a chimaera that showed a large reduction in CGRP potency (RAMP\textsubscript{386-89}). There was a tendency for a reduced rAmy potency for five of six chimaeras, however, it was only statistically significant for RAMP\textsubscript{350-53} and RAMP\textsubscript{386-89} (Figure 3.31 and Table 3.1).
Figure 3.31 cAMP data for rAmy responses at (a) RAMP\textsubscript{42-45}, (b) RAMP\textsubscript{50-53}, (c) RAMP\textsubscript{58-61}, (d) RAMP\textsubscript{70-73}, (e) RAMP\textsubscript{86-89} and (f) RAMP\textsubscript{94-97} AMY\textsubscript{1(a)} vs WT AMY\textsubscript{1(a)}. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
**Table 3.1** Summary of cAMP data for rAmy responses at the AMY$_{1(a)}$ receptors containing RAMP1/3 chimaeras, n=3. **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA followed by Dunnett’s test (one-way ANOVA was used for analysing these data as all the six chimaeras were examined in the same experiments with the same WT RAMP1 as control).**

<table>
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<th>Construct</th>
<th>pEC$_{50}$</th>
<th>E$_{\text{max}}$</th>
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<td>83.9 ± 1.44</td>
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<tr>
<td>RAMP3$_{70-73}$</td>
<td>10.6 ± 0.14</td>
<td>87.3 ± 3.51</td>
</tr>
<tr>
<td>RAMP3$_{86-89}$</td>
<td>8.99 ± 0.02</td>
<td>77.5 ± 2.23</td>
</tr>
<tr>
<td>RAMP3$_{94-97}$</td>
<td>10.0 ± 0.10</td>
<td>77.7 ± 1.23</td>
</tr>
</tbody>
</table>
3.3 Discussion

In total, 26 RAMP1/3 chimaeras were characterised with CT(a) in this chapter. Regions involved cover the entire N-terminal domain of RAMP1 which has been demonstrated to be the major determinant of receptor pharmacology. Tyr\(^\alpha\)h\(\alpha\)CGRP and h\(\beta\)CGRP were chosen to functionally characterise these RAMP1/3 chimeras with CT(a) because they showed the strongest discrimination between AMY\(_{(1)(a)}\) than AMY\(_{3(a)}\) in a previous study (Hay et al., 2005). A large number of chimaeras induced significant reduction in Tyr\(^\alpha\)h\(\alpha\)CGRP and h\(\beta\)CGRP potencies at the AMY\(_{(1)(a)}\) receptors (Table 3.2). Amongst them, only RAMP3\(_{18-21}\), RAMP3\(_{26-29}\), RAMP3\(_{42-44}\), RAMP3\(_{70-73}\) and the last four chimaeras which are close to or within the TM region did not show significantly altered CGRP responses. AMY\(_{(1)(a)}\) receptors containing mutants RAMP3\(_{22-25}\), RAMP3\(_{30-33}\), RAMP3\(_{34-47}\), RAMP3\(_{38-41}\), RAMP3\(_{62-65}\), RAMP3\(_{86-89}\), RAMP3\(_{90-93}\) displayed large reductions in potency for both Tyr\(^\alpha\)h\(\alpha\)CGRP and h\(\beta\)CGRP (>100-fold) (Table 3.2). That larger effects were observed with the chimaeras at the N-terminal part compared to those near the TM domain emphasised on the importance of the N-terminal domain of the RAMP for receptor pharmacology.

As expected, effects seen on Tyr\(^\alpha\)h\(\alpha\)CGRP and h\(\beta\)CGRP potencies were generally consistent; chimaeras showed reductions in Tyr\(^\alpha\)h\(\alpha\)CGRP potency also reduced h\(\beta\)CGRP potency, though the magnitude of reduction tends to be smaller for h\(\beta\)CGRP than Tyr\(^\alpha\)h\(\alpha\)CGRP for some mutants. On the other hand, the fact that a large number of chimaeras altered peptide potencies could not be possibly explained by the limited number of chimaeras that showed altered CLR expression. The RAMP1/CT\(_{(a)}\) complex seems more sensitive to structural changes introduced in RAMP1 than the RAMP1/CLR complex. It is possible that wider areas are involved in the RAMP1 to associate with CT\(_{(a)}\) than those involved in CLR association.
Table 3.2 Potency changes for hβCGRP and Tyr1αCGRP at RAMP1/3 chimaeras co-expressed with CT(a). The chimaeras at the N terminus are grouped based on their positions in the crystal structure of the RAMP1 N-terminal domain. NS, not significant, *p < 0.05, **p<0.01, ***p < 0.001 vs, WT AMY1(a) by unpaired t-test.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Tyr1αCGRP</th>
<th>hβCGRP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Significance</td>
<td>Fold change</td>
</tr>
<tr>
<td>RAMP318-21</td>
<td>NS</td>
<td>↔ 1</td>
</tr>
<tr>
<td>RAMP322-25</td>
<td>***</td>
<td>↓ 115</td>
</tr>
<tr>
<td>RAMP326-29</td>
<td>NS</td>
<td>↓ 9</td>
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<tr>
<td>RAMP330-33</td>
<td>***</td>
<td>↓ 214</td>
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<tr>
<td>RAMP334-37</td>
<td>***</td>
<td>↓ 148</td>
</tr>
<tr>
<td>RAMP338-41</td>
<td>***</td>
<td>↓ 427</td>
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<td>RAMP342-45</td>
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<td>↓ 4</td>
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<tr>
<td>RAMP346-49</td>
<td>***</td>
<td>↓ 35</td>
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<tr>
<td>RAMP350-53</td>
<td>***</td>
<td>↓ 12</td>
</tr>
<tr>
<td>RAMP354-57</td>
<td>***</td>
<td>↓ 30</td>
</tr>
<tr>
<td></td>
<td>Helix 1 and loop 1</td>
<td></td>
</tr>
<tr>
<td>RAMP358-61</td>
<td>**</td>
<td>↓ 6</td>
</tr>
<tr>
<td>RAMP362-65</td>
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<td>↓ 200</td>
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<tr>
<td>RAMP366-69</td>
<td>***</td>
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<tr>
<td>RAMP374-77</td>
<td>*</td>
<td>↓ 6</td>
</tr>
<tr>
<td>RAMP378-81</td>
<td>***</td>
<td>↓ 15</td>
</tr>
<tr>
<td>RAMP382-85</td>
<td>*</td>
<td>↓ 6</td>
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<tr>
<td></td>
<td>Helix 2 and loop 2</td>
<td></td>
</tr>
<tr>
<td>RAMP386-89</td>
<td>***</td>
<td>↓ 209</td>
</tr>
<tr>
<td>RAMP390-93</td>
<td>***</td>
<td>↓ 372</td>
</tr>
<tr>
<td>RAMP394-97</td>
<td>**</td>
<td>↓ 11</td>
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<tr>
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<td>↓ 13</td>
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<tr>
<td></td>
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<tr>
<td>RAMP3102-105</td>
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<td>NS</td>
<td>↓ 2</td>
</tr>
<tr>
<td>RAMP3118-121</td>
<td>*</td>
<td>↑ 3</td>
</tr>
</tbody>
</table>
Alterations in receptor cell-surface expression can affect peptide potencies in the transfected cells. An increase in receptor cell-surface expression could lead to an enhanced peptide potency whereas a reduced receptor cell-surface expression could weaken peptide potency (Bailey & Hay, 2007). As rAmy is equally potent at the AMY$_{1(a)}$ and AMY$_{3(a)}$ receptors, any change in rAmy potency at the mutant AMY$_{1(a)}$ receptors containing the chimaeras would reflect the change in their cell-surface expression. Thus, rAmy potency could be used as a surrogate to provide an indication for the cell-surface expression of the mutant AMY$_{1(a)}$ receptors. rAmy response was determined at the AMY$_{1(a)}$ receptors containing the six selected chimaeras. RAMP$_{350-53}$ and RAMP$_{386-89}$ which induced moderate to large reductions in CGRP potencies also reduced rAmy potency. Of note, the reduced rAmy potency observed with these two mutants did not correlate with the receptor expression data generated with CLR as CLR expression was not significantly affected by either mutant. Mutants RAMP$_{342-45}$ and RAMP$_{370-73}$ which had no significant effect on CGRP potency and mutants RAMP$_{38-61}$ and RAMP$_{94-97}$ which showed small to moderate effects in CGRP potency did not introduce any significant change to rAmy potency. Therefore, it is possible that small changes observed in peptide potency (~10 or < 10) are more likely to reflect changes in receptor function, whereas larger changes seen in peptide potency might result from reduced receptor expression at the cell surface.

In addition, the small RAMP1/RAMP3 chimaeras were also characterised with CLR in the CGRP receptors, these characterisations were performed by a collaborating group in Aston University (UK). In contrast to CT$_{(a)}$, less effects were seen with CLR; only six chimaeras induced significant reductions in hαCGRP potency, five of which were paralleled by a reduction in cell-surface expression (Qi et al., 2010). Although CGRP has high affinity and potency at both CGRP and AMY$_{1(a)}$ receptors, the substantial differences observed in the data generated with these small chimaeras in complex with CLR and CT$_{(a)}$ indicate that the structure-function relationship might be significantly different for RAMP1 in these two receptor complexes. These data are discussed and compared in Chapter 8.
Chapter 4

Characterisation of the effect of incorporating RAMP3 residues into RAMP1 on CGRP and AMY$_{1(a)}$ receptor function

4.1 Introduction

Given the long extracellular domains of the receptor components and the large endogenous peptide ligands, the chimaera approach seemed to be useful to perform a screen in the N-terminal domain of RAMP to narrow down residues of importance for peptide-receptor interactions to smaller regions. However, a large number of chimaeras induced significant non-specific changes to peptide potency which has made it difficult to interpret the data generated in Chapter 3. Therefore, strategic substitutions were employed in this chapter to explore the molecular basis for both CGRP and AMY$_{1(a)}$ receptor pharmacology.

Various deletion/chimaera mutations or alanine substitutions have been generated in the N-terminus of RAMP, however, little success has been made in identifying specific residues that are involved in ligand to receptor interaction. In addition, it is very challenging to confirm whether a particular residue of RAMP is directly involved in ligand to receptor interaction or the presence of the residue actually introduces conformational changes to the receptor for ligand to bind. The crystal structure of the RAMP1 N terminus has suggested some residues that are likely to form part of the binding pocket for CGRP (Kusano et al., 2008), nevertheless, alanine substitutions at these residues suggested that they had little effect on CGRP potency, except for W84A RAMP1 which was paralleled by a reduced receptor expression at the cell surface (Moore et al., 2010). On the other hand, a residue in RAMP1, W74, has been identified to play a key role in the species selectivity for the very high-affinity binding of the CGRP antagonist BIBN4096BS to the human CGRP receptors (Hay et al., 2003; Mallee et al., 2002; Salvatore et al., 2006). Furthermore, BIBN4096BS showed a high-affinity binding to the RAMP1 containing CGRP receptor, but not the RAMP3 containing AM$_2$ receptor. However, when the native Glu at position 74 in RAMP3 was mutated to Trp (E74W), the mutant AM$_2$ receptors showed more sensitivity to BIBN4096BS (Hay et al., 2006a). A similar observation was also seen at the AMY$_{3(a)}$ receptor containing the E74W mutation in RAMP3. This study also reported an interesting observation in the peptide agonist responses, where the E74W RAMP3 mutant induced selective reduction in hAM, but not CGRP potency at the AM$_2$ receptor (Hay et al., 2006a). The study has suggested an
important and differential role that residue E74 plays in the receptor to AM and CGRP interactions.

Sequence alignment of human RAMPs reveals that RAMP2 also has Glu at the equivalent position (E101) to E74 in RAMP3 (Figure 4.1). Eight residues including E74 that are conserved in RAMPs 2 and 3 but different in RAMP1 in the N termini of human RAMPs (beyond the predicted signal peptides) were identified (Figure 4.1 and 4.2). The association of RAMP1 with CLR results in a CGRP receptor, whilst AM receptors are formed when RAMP2 or RAMP3 complexes with CLR. The conservation of these eight residues in RAMPs 2 and 3 but not RAMP1 suggests an important role of these residues in conferring peptide selectivity of AM over CGRP. Therefore, residues at these eight positions were swapped between RAMP1 and RAMP3 using SDM. It was hypothesised that these residues were responsible for conferring pharmacological selectivity to the CGRP (RAMP1/CLR) and AM receptors (RAMP2/CLR or RAMP3/CLR). The RAMP1 mutants containing these eight point mutations were characterised with CLR and data generated are presented in this Chapter.

In addition, as introduced in Chapter 3, CGRP displays different potencies at AMY$_{1(a)}$ and AMY$_{3(a)}$, thus it is possible that the sequence differences at these eight positions may be potentially important for determining CGRP potency at AMY$_{1(a)}$. Therefore, these eight RAMP1 mutants were also characterised with CT$_{(a)}$ in this chapter. The reciprocal RAMP3 mutants with the RAMP1 residues introduced at these eight positions were also generated and characterised with both CLR and CT$_{(a)}$; the data are presented in Chapter 5.
Figure 4.1 Amino acid sequence alignment of three human RAMPs. The predicted signal peptide is shown in blue and the TM domain is shown in red. The residues that are conserved between RAMP2 and RAMP3 but different in RAMP1 at the N-terminal domain (beyond the signal peptide) are highlighted in green.

Figure 4.2 (a) The RAMP1 crystal structure (Kusano et al., 2008) and (b) the RAMP3 model (Bailey et al., 2010) of the N-terminal domain. The residues that have been mutated in Chapters 4 and 5 are coloured in red.
4.2 Results

4.2.1 mycRAMP1 mutants with HA-CLR; CGRP receptor

4.2.1.1 Cell-surface expression

The CGRP receptor expression was estimated by measuring HA-CLR expression. The cell surface expression of CLR in complex with the RAMP1 mutants is shown in Figure 4.3. Compared to CLR alone, the cotransfection of RAMP1 induced a significant increase in CLR cell-surface expression ($p < 0.001$ by one-way ANOVA followed by Dunnett’s test). The CLR expression was equivalent in all the CGRP receptors containing the RAMP1 mutants (analysed by one-way ANOVA, followed by Dunnett’s test).

![Figure 4.3](image-url)

**Figure 4.3** ELISA data for mycRAMP1 mutants expressed with HA-CLR, measuring HA-CLR expression at the cell surface. Data are mean ± s.e.m. of three to six independent experiments, each performed with eight replicates. Data were analysed by one-way ANOVA, followed by Dunnett’s test. Significance was achieved at $p < 0.05$. None of mutant receptor expression was significantly different from the WT receptor expression.
4.2.1.2 Functional characterisation using cAMP assays

The CGRP receptor and AM<sub>2</sub> receptor phenotypes can be pharmacologically differentiated by their differential peptide selectivity. hαCGRP was ~2400-fold more potent at the CGRP receptor compared to the AM<sub>2</sub> receptor (Figure 4.4a), whereas hAM was ~20-fold more potent at the AM<sub>2</sub> receptor compared to the CGRP receptor (Figure 4.4b). The hαCGRP responses were pEC<sub>50</sub> ± s.e.m., CGRP 10.6 ± 0.19 vs AM<sub>2</sub> 7.21 ± 0.08 (p < 0.01 by unpaired t-test, n=4) and E<sub>max</sub> ± s.e.m., CGRP 70.5 ± 3.50 vs AM<sub>2</sub> 65.7 ± 4.32. The AM responses were pEC<sub>50</sub> ± s.e.m., CGRP 8.69 ± 0.16 vs AM<sub>2</sub> 9.96 ± 0.09 (p < 0.01 by unpaired t-test, n=4) and E<sub>max</sub> ± s.e.m., CGRP 72.7 ± 3.94 vs AM<sub>2</sub> 64.2 ± 1.99. Therefore, the mutant CGRP receptor function was investigated for both peptides. Based on the hypothesis, the RAMP1 mutants containing the RAMP3 residues were expected to reduce hαCGRP potency but enhance hAM potency in the CGRP receptors, if the residues were important for the peptide to receptor interactions.

![Graphs showing cAMP responses](image)

**Figure 4.4** cAMP data for (a) hαCGRP and (b) hAM responses at the WT CGRP and WT AM<sub>2</sub> (RAMP3/CLR) receptors. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.1.2.1 A34E CGRP receptor

hαCGRP potency was significantly enhanced at the mutant A34E CGRP receptor compared to the WT receptor (Figure 4.5a). pEC$_{50}$ for hαCGRP at the mutant receptor was 10.6 ± 0.07 compared to 10.2 ± 0.07 at the WT receptor (pEC$_{50}$ ± s.e.m., p < 0.05 by unpaired t-test, n=3), displaying a ~3-fold increase in the potency. E$_{\text{max}}$ was also significantly enhanced; 80.3 ± 0.96 for the mutant receptor and 74.5 ± 0.27 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m., p < 0.01 by unpaired t-test, n=3). On the other hand, hAM responses were not significantly altered: pEC$_{50}$ ± s.e.m., mutant 8.21 ± 0.07 vs WT 8.27 ± 0.19 and E$_{\text{max}}$ ± s.e.m., mutant 71.6 ± 0.85 vs WT 68.7 ± 5.84 (n=3) (Figure 4.5b).

**Figure 4.5** cAMP data for (a) hαCGRP and (b) hAM responses at the mutant A34E CGRP receptor and the WT CGRP receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.1.2.2 V46D CGRP receptor

There was no significant difference in peptide response between the mutant V46D CGRP receptor and WT receptor for either hαCGRP or hAM (Figure 4.6). pEC\textsubscript{50} for hαCGRP at the mutant receptor were 10.6 ± 0.13 compared to 10.2 ± 0.11 for the WT receptor (pEC\textsubscript{50} ± s.e.m.; n=3). The E\textsubscript{max} values were 85.5 ± 4.13 for the mutant receptor and 78.7 ± 3.61 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m.). hAM responses were: pEC\textsubscript{50} ± s.e.m., mutant 8.32 ± 0.10 vs WT 8.50 ± 0.16 and E\textsubscript{max} ± s.e.m., mutant 74.8 ± 2.78 vs WT 77.0 ± 1.48 (n=3).

Figure 4.6 cAMP data for (a) hαCGRP and (b) hAM responses at the mutant V46D CGRP receptor and the WT CGRP receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.1.2.3 W74E CGRP receptor

There was no difference in peptide response between the mutant W74E CGRP receptor and the WT receptor for hαCGRP: pEC\textsubscript{50} ± s.e.m., mutant 10.6 ± 0.20 vs WT 10.5 ± 0.20 and E\textsubscript{max} ± s.e.m., mutant 77.4 ± 3.75 vs WT 75.8 ± 3.88 (n=5) (Figure 4.7a). On the other hand, hAM potency was significantly enhanced at the mutant W74E CGRP receptor compared to the WT receptor. pEC\textsubscript{50} for hαCGRP at the mutant receptor was 9.26 ± 0.08 compared to 8.43 ± 0.15 at the WT receptor (pEC\textsubscript{50} ± s.e.m., \(p < 0.01\) by unpaired t-test, n=5), displaying a ~7-fold increase in the potency. E\textsubscript{max} was not significantly altered as 74.9 ± 3.20 for the mutant receptor and 70.4 ± 2.65 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m.) (Figure 4.7b).

![Figure 4.7](image_url)

**Figure 4.7** cAMP data for (a) hαCGRP and (b) hAM responses at the mutant W74E CGRP receptor and the WT CGRP receptor. The graphs are representative of five independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.1.2.4 A87P CGRP receptor

There was no significant difference in peptide response between the mutant A87P CGRP receptor and the WT receptor for either hαCGRP or hAM (Figure 4.8). pEC$_{50}$ for hαCGRP at the mutant receptor were 10.6 ± 0.25 compared to 10.2 ± 0.16 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The E$_{max}$ values were 85.7 ± 5.13 for the mutant receptor and 80.3 ± 2.79 for the WT receptor, respectively (E$_{max}$ ± s.e.m.). hAM responses were: pEC$_{50}$ ± s.e.m., mutant 8.41 ± 0.09 vs WT 8.50 ± 0.15 and E$_{max}$ ± s.e.m., mutant 76.3 ± 2.85 vs WT 74.7 ± 1.17 (n=3).

Figure 4.8 cAMP data for (a) hαCGRP and (b) hAM responses at the mutant A87P CGRP receptor and the WT CGRP receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.1.2.5 E88L CGRP receptor

There was no significant difference in peptide response between the mutant E88L CGRP receptor and the WT receptor for either hαCGRP or hAM (Figure 4.9). pEC$_{50}$ for hαCGRP at the mutant receptor were 10.3 ± 0.29 compared to 10.3 ± 0.14 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The E$_{\text{max}}$ values were 75.6 ± 0.85 for the mutant receptor and 79.4 ± 2.31 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). hAM responses were: pEC$_{50}$ ± s.e.m., mutant 8.48 ± 0.06 vs WT 8.68 ± 0.16 and E$_{\text{max}}$ ± s.e.m., mutant 72.1 ± 3.68 vs WT 70.1 ± 5.29 (n=3).

![Graphs showing cAMP data for (a) hαCGRP and (b) hAM responses at the mutant E88L CGRP receptor and the WT CGRP receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.](image-url)

**Figure 4.9** cAMP data for (a) hαCGRP and (b) hAM responses at the mutant E88L CGRP receptor and the WT CGRP receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.1.2.6 V89A CGRP receptor

There was no significant difference in peptide response between the mutant V89A CGRP receptor and the WT receptor for either hαCGRP or hAM (Figure 4.10). pEC$_{50}$ for hαCGRP at the mutant receptor were 9.62 ± 0.19 compared to 9.86 ± 0.18 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The E$_{\text{max}}$ values were 75.5 ± 1.93 for the mutant receptor and 73.1 ± 5.58 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). hAM responses were: pEC$_{50}$ ± s.e.m., mutant 7.70 ± 0.15 vs WT 7.81 ± 0.43 and E$_{\text{max}}$ ± s.e.m., mutant 76.0 ± 3.02 vs WT 72.4 ± 3.25 (n=3).

**Figure 4.10** cAMP data for (a) hαCGRP and (b) hAM responses at the mutant V89A CGRP receptor and the WT CGRP receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.1.2.7 F93I CGRP receptor

hαCGRP potency was significantly reduced at the mutant F93I CGRP receptor compared to the WT receptor (Figure 4.11a). pEC$_{50}$ for hαCGRP at the mutant receptor was 9.40 ± 0.12 compared to 10.1 ± 0.15 at the WT receptor (pEC$_{50}$ ± s.e.m., $p < 0.05$ by unpaired t-test, n=3), displaying a ~5-fold reduction in potency. E$_{\text{max}}$ was not significantly affected as 66.9 ± 4.98 for the mutant receptor and 72.2 ± 4.79 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). On the other hand, hAM response was not significantly altered: pEC$_{50}$ ± s.e.m., mutant 7.60 ± 0.18 vs WT 8.03 ± 0.25 and E$_{\text{max}}$ ± s.e.m., mutant 73.1 ± 4.35 vs WT 70.4 ± 2.08 (n=3) (Figure 4.11b).

**Figure 4.11** cAMP data for (a) hαCGRP and (b) hAM responses at the mutant F93I CGRP receptor and the WT CGRP receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.1.2.8 S103N CGRP receptor

There was no significant difference in peptide response between the mutant S103N CGRP receptor and the WT receptors for either hαCGRP or hAM (Figure 4.12). pEC$_{50}$ for hαCGRP at the mutant receptor were 10.5 ± 0.16 compared to 10.1 ± 0.25 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The E$_{\text{max}}$ values were 73.9 ± 3.78 for the mutant receptor and 79.3 ± 3.43 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). hAM responses were: pEC$_{50}$ ± s.e.m., mutant 8.20 ± 0.16 vs WT 8.23 ± 0.29 and E$_{\text{max}}$ ± s.e.m., mutant 69.9 ± 2.20 vs WT 69.8 ± 3.33 (n=3).

![Figure 4.12](image)

**Figure 4.12** cAMP data for (a) hαCGRP and (b) hAM responses at the mutant S103N CGRP receptor and the WT CGRP receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.1.2.9 Characterisation of triple mutant RAMP3\textsubscript{86-89} CGRP receptor

Sequence alignment shows that there are three consecutive residues at positions 87-89 that are conserved between RAMPs 2 and 3 but different in RAMP1 (Figure 4.1). Individual substitutions at these three positions in RAMP1 did not induce any significant effect to the CGRP receptor function (Sections 4.2.1.2.4-4.2.1.2.6). However, it is possible that the residues at this region may together have a role in receptor function. This triplet mutant was identified in one of the RAMP1/3 chimaeras (Chapter 3); RAMP3\textsubscript{86-89}, which has a conserved N86 between RAMPs 1 and 3 and the three divergent residues at position 87-89 replaced by those from RAMP3. RAMP3\textsubscript{86-89} has been characterised with CT\textsubscript{(a)} in Chapter 3; characterisation with CLR was in addition performed in this chapter to study role of region 86-89 in RAMP1 to CGRP receptor function.

As illustrated in Chapter 3, ELISA data showed that cell surface expression of the mutant RAMP3\textsubscript{86-89} CGRP receptor was not significantly different from that of the WT receptor (Figure 4.13a). On the other hand, both hαCGRP and hAM potencies were significantly reduced at the mutant RAMP3\textsubscript{86-89} CGRP receptor compared to the WT receptor (Figure 4.13b&c). pEC\textsubscript{50} for hαCGRP at the mutant was 9.45 ± 0.22 compared to 10.6 ± 0.19 at the WT receptor (pEC\textsubscript{50} ± s.e.m., p < 0.01 by unpaired t-test, n=4), displaying a ~14-fold reduction in potency. E\textsubscript{max} was not significantly altered; 63.8 ± 2.65 for the mutant receptor and 70.5 ± 3.51 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m.). Furthermore, there was a ~11-fold reduction in hAM potency (pEC\textsubscript{50} ± s.e.m., mutant 7.63 ± 0.06 vs WT 8.69 ± 0.16, p < 0.001 by unpaired t-test, n=4) whereas the maximum response was not significantly affected (E\textsubscript{max} ± s.e.m., mutant 71.6 ± 1.55 vs WT 72.7 ± 3.94). As both hαCGRP and hAM potency was reduced by RAMP3\textsubscript{86-89}, it seemed that the effect might be non-specific. Therefore, peptide response of hβCGRP was determined at the mutant CGRP receptor containing RAMP3\textsubscript{86-89} (Figure 4.13d). Its response was not significantly altered: pEC\textsubscript{50} ± s.e.m., 10.7 ± 0.04 vs WT 10.9 ± 0.16 and E\textsubscript{max} ± s.e.m., mutant 71.56 ± 2.74 vs WT 67.44 ± 6.44 (n=4) (Figure 4.13d).
Figure 4.13 (a) ELISA data for RAMP3_86-89 expressed with HA-CLR, measuring HA-CLR expression at the cell surface. Data are mean ± s.e.m. of three independent experiments, each performed with eight replicates. (b) cAMP data for hαCGRP, (c) hAM and (d) hβCGRP responses at RAMP3_86-89 CGRP and WT CGRP. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
### 4.2.2 mycRAMP1 mutants with HA-CT<sub>(a)</sub>; AMY<sub>1(a)</sub> receptor

#### 4.2.2.1 Cell-surface expression

As CT<sub>(a)</sub> has significant expression and acts as a functional receptor alone at the cell surface, the cell-surface expression of AMY<sub>1(a)</sub> receptor phenotype was determined by measuring mycRAMP1 expression. The cell-surface expression of mycRAMP1 can be used as an indication of RAMP1 and CT<sub>(a)</sub> association because RAMP1 does not have significant expression at the cell surface in the absence of CT<sub>(a)</sub>.

Several mutations introduced into mycRAMP1 had a significant impact on the receptor cell-surface expression (Figure 4.14). A34E, E88L, V89A and F93I all induced significant reductions in mycRAMP1 expression in complex with CT<sub>(a)</sub> at the cell surface, whereas mycRAMP1 expression was enhanced in the mutant receptors containing W74E and S103N. The expression of the other mutant receptors was not altered compared to the WT receptor.

![ELISA data for mycRAMP1 mutants expressed with HA-CT<sub>(a)</sub>](image)

**Figure 4.14** ELISA data for mycRAMP1 mutants expressed with HA-CT<sub>(a)</sub>, measuring mycRAMP1 expression at the cell surface. **p < 0.01** vs WT by one-way ANOVA followed by Dunnett’s test. Data are mean ± s.e.m. of three independent experiments, each performed with eight replicates.
### 4.2.2.2 Functional characterisation using cAMP assays

rAmy and hβCGRP were chosen for characterising the eight RAMP1 mutants containing the RAMP3 residues with HA-CT\(_{(a)}\) in the AMY\(_{1(a)}\) receptor. As demonstrated in Chapter 3, rAmy is the cognate peptide that displays high and equivalent potency at both AMY\(_{1(a)}\) and AMY\(_{3(a)}\) whereas hβCGRP, also a high potency agonist, shows higher potency at AMY\(_{1(a)}\) than AMY\(_{3(a)}\). Therefore, based on the hypothesis, it was expected to see reduced hβCGRP potency but unaffected rAmy potency at the AMY\(_{1(a)}\) receptors containing these RAMP1 mutants, if the residues were important for hβCGRP interactions.

#### 4.2.2.2.1 A34E AMY\(_{1(a)}\) receptor

There was no significant difference in peptide response observed between the mutant A34E AMY\(_{1(a)}\) and the WT receptor for either rAmy or hβCGRP (Figure 4.15). pEC\(_{50}\) for rAmy at the mutant receptor were 10.8 ± 0.17 compared with 10.4 ± 0.12 at the WT receptor (pEC\(_{50}\) ± s.e.m.; n=3). The E\(_{\text{max}}\) values were 80.1 ± 1.31 for the mutant receptor and 75.5 ± 4.52 for the WT receptor, respectively (E\(_{\text{max}}\) ± s.e.m.). hβCGRP responses were: pEC\(_{50}\) ± s.e.m., mutant 10.9 ± 0.18 vs WT 10.7 ± 0.22 and E\(_{\text{max}}\) ± s.e.m., mutant 79.1 ± 2.77 vs WT 71.6 ± 9.67 (n=3).

![Figure 4.15](image-url)

**Figure 4.15** cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant A34E AMY\(_{1(a)}\) receptor and the WT AMY\(_{1(a)}\) receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.2.2 V46D AMY<sub>1(a)</sub> receptor

There was no significant difference in peptide response observed between the mutant V46D AMY<sub>1(a)</sub> receptor and the WT receptor for either rAmy or hβCGRP (Figure 4.16). pEC<sub>50</sub> for rAmy response at the mutant receptor were 10.8 ± 0.13 compared to 10.5 ± 0.11 at the WT receptor (pEC<sub>50</sub> ± s.e.m.; n=3). The E<sub>max</sub> values were 73.9 ± 7.21 for the mutant receptor and 74.0 ± 0.81 for the WT receptor, respectively (E<sub>max</sub> ± s.e.m.). hβCGRP responses were: pEC<sub>50</sub> ± s.e.m., mutant 10.8 ± 0.12 vs WT 10.5 ± 0.11 and E<sub>max</sub> ± s.e.m., mutant 72.7 ± 3.95 vs WT 68.9 ± 7.43 (n=3).

**Figure 4.16** cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant V46D AMY<sub>1(a)</sub> receptor and the WT AMY<sub>1(a)</sub> receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
Chapter 4

4.2.2.2.3 W74E AMY₁(a) receptor

rAmy potency was significantly enhanced at the mutant W74E AMY₁(a) receptor compared to the WT receptor (Figure 4.17a). pEC₅₀ for rAmy at the mutant receptor was 10.9 ± 0.07 compared to 10.2 ± 0.15 at the WT receptor (pEC₅₀ ± s.e.m., p < 0.05 by unpaired t-test, n=3), displaying a ~5-fold increase in the potency. Eₘₐₓ was not significantly affected as 73.1 ± 2.71 for the mutant receptor and 77.7 ± 3.36 for the WT receptor, respectively (Eₘₐₓ ± s.e.m.). On the other hand, hβCGRP response was not significantly altered: pEC₅₀ ± s.e.m., mutant 11.0 ± 0.18 vs WT 10.7 ± 0.15 and Eₘₐₓ ± s.e.m., mutant 80.0 ± 3.41 vs WT 83.0 ± 5.20 (n=3) (Figure 4.17b).

![Graphs showing cAMP data for rAmy and hβCGRP responses](image)

**Figure 4.17** cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant W74E AMY₁(a) receptor and the WT AMY₁(a) receptor. The graphs are representative of three to four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.2.2.4 A87P AMY$_{1(a)}$ receptor

There was no significant difference in peptide response observed between the mutant A87P AMY$_{1(a)}$ receptor and the WT receptor for either rAmy or hβCGRP (Figure 4.18). pEC$_{50}$ for rAmy at the mutant receptor were 10.8 ± 0.16 compared to 10.2 ± 0.16 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The E$_{\text{max}}$ values were 81.2 ± 3.15 for the mutant receptor and 75.6 ± 3.76 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). hβCGRP responses were: pEC$_{50}$ ± s.e.m., mutant 11.1 ± 0.09 vs WT 10.9 ± 0.04 and E$_{\text{max}}$ ± s.e.m., mutant 73.2 ± 4.65 vs WT 69.1 ± 6.94 (n=3).

Figure 4.18 cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant A87P AMY$_{1(a)}$ receptor and the WT AMY$_{1(a)}$ receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.2.2.5 E88L AMY₁(a) receptor

There was no difference in peptide response between the mutant E88L AMY₁(a) receptor and the WT receptor for rAmy: pEC₅₀ ± s.e.m., mutant 9.96 ± 0.18 vs WT 10.2 ± 0.13 and Eₘₐₓ ± s.e.m., mutant 73.3 ± 3.68 vs WT 69.8 ± 9.61 (n=3) (Figure 4.19a). On the other hand, hβCGRP was significantly reduced at the mutant E88L AMY₁(a) receptor compared to the WT receptor. pEC₅₀ for hβCGRP at the mutant receptor was 10.4 ± 0.15 compared to 10.9 ± 0.03 for the WT receptor (pEC₅₀ ± s.e.m., p < 0.05 by unpaired t-test, n=3), displaying a ~3-fold reduction in the potency. Eₘₐₓ was not significantly altered as 80.8 ± 3.40 for the mutant receptor and 79.8 ± 4.13 for the WT receptor, respectively (Eₘₐₓ ± s.e.m.) (Figure 4.19b).

Figure 4.19 cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant E88L AMY₁(a) receptor and the WT AMY₁(a) receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
Both rAmy and hβCGRP potencies were significantly reduced at the mutant V89A AMY\textsubscript{1(a)} receptor compared to the WT receptor (Figure 4.20). pEC\textsubscript{50} for rAmy at the mutant receptor was 9.71 ± 0.02 compared to 10.2 ± 0.15 at the WT receptor (pEC\textsubscript{50} ± s.e.m., \( p < 0.05 \) by unpaired t-test, \( n=3 \)), displaying a ~3-fold reduction in potency. \( E_{\text{max}} \) was not significantly altered; 76.9 ± 12.4 for the mutant receptor and 63.7 ± 6.69 for the WT receptor, respectively (\( E_{\text{max}} \) ± s.e.m.). There was a ~7-fold reduction in hβCGRP potency (pEC\textsubscript{50} ± s.e.m., mutant 9.86 ± 0.09 vs WT 10.7 ± 0.13, \( p < 0.01 \) by unpaired t-test, \( n=3 \)) whereas the maximum response was not significantly affected (\( E_{\text{max}} \) ± s.e.m., mutant 82.3 ± 5.62 vs WT 68.8 ± 2.48).

**Figure 4.20** cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant V89A AMY\textsubscript{1(a)} receptor and the WT AMY\textsubscript{1(a)} receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.2.2.7 F93I AMY1(a) receptor

Both rAmy and hβCGRP potencies were significantly reduced at the mutant F93I AMY1(a) receptor compared to the WT receptor (Figure 4.21). pEC$_{50}$ for rAmy at the mutant receptor was $9.08 \pm 0.28$ compared to $10.3 \pm 0.28$ at the WT receptor (pEC$_{50}$ s.e.m., $p < 0.05$ by unpaired t-test, n=3), displaying a ~17-fold reduction in potency. E$_{\text{max}}$ was not significantly altered; $79.8 \pm 4.67$ for the mutant receptor and $74.9 \pm 2.35$ for the WT receptor, respectively (E$_{\text{max}}$ s.e.m.). There was a ~46-fold reduction in hβCGRP potency (pEC$_{50}$ ± s.e.m., mutant 9.04 ± 0.08 vs WT 10.7 ± 0.16, $p < 0.001$ by unpaired t-test, n=3) whereas the maximum response was not significantly affected (E$_{\text{max}}$ ± s.e.m., mutant 68.7 ± 2.35 vs WT 69.3 ± 7.26).

**Figure 4.21** cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant F93I AMY1(a) receptor and the WT AMY1(a) receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.2.2.2.8 S103N AMY₁(a) receptor

rAmy potency was significantly enhanced at the mutant S103N AMY₁(a) receptor compared to the WT receptor (Figure 4.22a). pEC$_{50}$ for rAmy at the mutant receptor was 10.9 ± 0.02 compared to 10.5 ± 0.11 at the WT receptor (pEC$_{50}$ ± s.e.m., p < 0.05 by unpaired t-test, n=3), displaying a small ~3-fold increase in the potency. E$_{\text{max}}$ was not significantly affected; 79.9 ± 5.49 for the mutant receptor and 73.8 ± 3.40 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). On the other hand, hβCGRP response was not significantly altered: pEC$_{50}$ ± s.e.m., mutant 10.9 ± 0.10 vs WT 10.7 ± 0.08 and E$_{\text{max}}$ ± s.e.m., mutant 76.3 ± 3.06 vs WT 73.4 ± 6.83 (n=3) (Figure 4.22b).

Figure 4.22 cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant S103N AMY₁(a) receptor and the WT AMY₁(a) receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
4.3 Discussion

In this chapter, it was hypothesised that residues conserved in RAMPs 2 and 3 but not RAMP1 contribute to the differential receptor pharmacologies possessed by the RAMP3-based receptors and RAMP1-based receptors. Based on this hypothesis, it was expected that RAMP1 mutants with RAMP3 residues incorporated would elicit a reduced CGRP potency and/or an enhanced AM potency when coexpressed with CLR, whereas reduced CGRP potencies were expected when coexpressed with CT(a). In total, eight mycRAMP1 mutants were studied and differential effects of the mutations were observed.

When expressed with CLR, all the mycRAMP1 mutants containing CGRP receptors showed normal cell-surface expression levels that were comparable with that of the WT receptor. Three mutations, A34E, F93I and W74E, caused alterations in either hAM or hαCGRP potency, suggesting the potential importance of these residues. Mutation F93I in this study selectively reduced hαCGRP potency in the absence of any change in hAM potency. On the other hand, F93A CGRP showed reductions in both CGRP binding and receptor expression but CGRP potency was not affected in a previous study (Kuwasako et al., 2003a). Thus, the effects at position 93 seemed to be substitution dependent. Although the crystal structure of the RAMP1 N terminus has suggested that F93 is one of the residues forming the CLR interaction interface (Kusano et al., 2008), current experimental data does not provide enough support to demonstrate the direct involvement of F93 in the CLR interactions.

A previous study has demonstrated that the E74W mutation in RAMP3 selectively reduced hAM potency at the AM2 receptor (Hay et al., 2006a), but exactly the opposite effect was observed with W74E RAMP1 when coexpressed with CLR in this thesis. hAM potency was significantly enhanced at the W74E CGRP receptor. This effect appeared to be specific to hAM as hαCGRP potency was not altered by W74E mutation. The reciprocal nature of the effect in hAM potency suggests that residues at position 74 may play an important role in AM interactions. On the other hand, the contribution of residues at position 74 to CGRP potency remains unclear although W74 has been identified on the crystal structure of the RAMP1 N terminus as one of the potential interacting residues for CGRP binding (Kusano et al., 2008) and has also been shown to be involved in the interactions of small molecule CGRP antagonists olcegepant and telcagepant with the CGRP receptor (Hay et al., 2006a; Koth et al., 2010; Mallee et al., 2002; Moore et al., 2010). W74 may not be directly involved in CGRP interactions with the receptor as alanine or lysine substitutions at this position did...
not affect CGRP potency (Hay et al., 2006a; Moore et al., 2010). Further investigations have been carried out at position 74 to elucidate the nature of the interaction at this position (Chapter 6).

The individual mutants A87P, E88L and V89A failed to elicit any effect on peptide response when coexpressed with CLR. To test the idea that these residues may have a role together, RAMP1 chimaera (RAMP3\textsubscript{86-89}) with all the three different residues replaced with the RAMP3 residues at positions 86-89 was in addition generated and analysed with CLR. RAMP3\textsubscript{86-89} chimaera reduced both hαCGRP and hAM potencies without affecting receptor cell-surface expression, suggesting that region 86-89 plays an important role in peptide potency. However, it was very surprising to see that the hβCGRP response was not affected by this mutation. There are only three residues that are different between hαCGRP and hβCGRP (refer to sequence alignment in Chapter 1, Figure 1.1), suggesting that region 86-89 in RAMP3 is very likely to be in close proximity to these three residues when associates with CLR. The differential effect seen with hαCGRP and hβCGRP also suggests that there are subtle differences in the binding pockets in the CGRP receptor for these two peptides. To further explore the role of these residues in RAMP3, the reciprocal RAMP3 triple mutant was generated and analysed with CLR in chapter 5.

In the characterisation with CT\textsubscript{(a)}, several mutants showed changes in either rAmy or hβCGRP potency or in both, however, most of them were paralleled by altered cell-surface expression. W74E mycRAMP1 and N103S mycRAMP1 both led to enhanced rAmy potency. This was paralleled by increased cell-surface expression of the mutant mycRAMP1s. The hβCGRP potency was also enhanced for W74E and N103S though statistical significance was not achieved. It is therefore more likely that the altered peptide potency was a result of higher cell-surface expression of these mutant AMY\textsubscript{1(a)} receptors. Similarly, both rAmy and hβCGRP potency was significantly reduced at the V89A and F93I containing mutant receptors which was accompanied by significant loss of cell-surface expression of the mutant receptor.

Interestingly, two mutations W74E and F93I affected potency of one or both peptides analysed in both CGRP and AMY\textsubscript{1(a)} receptors. Although the changes in peptide potency at the AMY\textsubscript{1(a)} receptors were accompanied by alterations in the receptor cell-surface expression whereas the CGRP receptor expression was not significantly affected, it may
imply a possible common role for these residues of RAMP1 in CLR/CT(a) association. However, the degree of involvement of the two residues may be different in the two receptors.

Changes seen in peptide potency could have resulted from altered peptide to receptor interactions caused by the mutations in the RAMP, but the mutations could also have affected receptor signalling, i.e. Gαs coupling for cAMP production, and thus altered potencies. However, the latter is less likely. For CLR, it has been suggested that RAMPs play little role in the CGRP or AM receptor-mediated signalling, presumably due to the presence of RCP (Udawela et al., 2006a). The scenario is different with CTR; the C-terminal domains of RAMPs have been shown to be involved in G protein coupling (Udawela et al., 2006a). The mutations introduced all locate in the N terminus of the RAMP and it is thus more likely to have an effect on the peptide to receptor interaction sites which are also mainly in the N-terminal domains of the receptor complex than the G protein coupling sites that sit at the intracellular domains. Nevertheless, the possibility that the mutations may have introduced some overall conformational changes to the receptor which affected receptor signalling can not be ruled out.

It is apparent that the RAMP1 mutants had greater impact on the cell-surface expression of the AMY1(a) receptors than the CGRP receptors. This difference seen in expression between the CGRP and AMY1(a) receptors was not likely caused by the different measurements of the receptor cell-surface expression by either HA-CLR expression or mycRAMP1 expression, as it has been demonstrated in Chapter 2 that the two measurements generally provided consistent estimation of the receptor complex expression at the cell surface. Therefore, despite the homology between CLR and CT(a), it is apparent that the RAMP1/CT(a) complex is more sensitive to the mutations introduced into RAMP1 than the RAMP1/CLR complex. The fact that the RAMP1 mutations had more effects on the receptor cell-surface expression in complex with CT(a) than with CLR may suggest that the RAMP1 and CT(a) interactions may be significantly weaker than the RAMP1 and CLR association. This is consistent with the observations from Chapter 3 where a majority of RAMP1/3 chimaeras affected peptide potencies in the AMY1(a) receptors.
Chapter 5

Characterisation of the effect of incorporating individual RAMP1 residues into RAMP3 on AM$_2$ and AMY$_{3(a)}$ receptor function

5.1 Introduction

As introduced in Chapter 4, eight positions were identified in human RAMPs at which the residues are conserved between RAMPs 2 and 3, but different in RAMP1 (refer to Chapter 4, Figure 4.1 for sequence alignment). It was hypothesised that these different amino acid residues contribute to differential receptor specific pharmacology displayed by the RAMP1-based receptors and the RAMP3-based receptors. Data generated from the characterisation of the eight RAMP1 mutants with the RAMP3 residues incorporated at these positions were presented in Chapter 4. Of the eight mutants characterised with CLR, two mutants showed selective alteration in peptide potency that was in line with the hypothesis; F93I RAMP1 reduced hαCGRP potency whereas W74E RAMP1 enhanced hAM potency in the absence of any significant change in the CGRP receptor expression at the cell surface. On the other hand, a number of RAMP1 mutants affected rAmy and hβCGRP potencies when coexpressed with CT$_{(a)}$, however, they were all paralleled by altered receptor cell-surface expression, suggesting that the RAMP1/CT$_{(a)}$ complex is more sensitive to residue changes than the RAMP/CLR complex. In this chapter, to study the role of the residues at the eight corresponding positions in RAMP3, the reciprocal RAMP3 mutants with the RAMP1 residues incorporated were generated. The mutations were made in an untagged version of human RAMP3, namely E35A, D46V, E74W, P87A, L88E, A89V, I93F and N103S. These include E74 which has been suggested to contribute to AM potency at the AM$_2$ receptor (Hay et al., 2006a). However, its role in peptide interactions to the AMY$_{3(a)}$ receptor has never been demonstrated.

Similar characterisations were performed with these RAMP3 mutants to those performed in Chapter 3, i.e. the mutants were characterised with both CLR in the AM$_2$ receptor and CT$_{(a)}$ in the AMY$_{3(a)}$ receptor, using peptides hAM and hαCGRP or rAmy and hβCGRP as pharmacological tools, respectively. It was hypothesised that the introduction of RAMP1 residues into RAMP3 would make AM$_2$ or AMY$_{3(a)}$ receptor more like CGRP or AMY$_{1(a)}$ receptor and therefore would display more CGRP receptor-like and AMY$_{1(a)}$ receptor-like
pharmacology, respectively. More specific hypotheses are explained in Sections 5.2.1.2 and 5.2.2.

5.2 Results

5.2.1 RAMP3 mutants with HA-CLR; AM₂ receptor

5.2.1.1 Cell-surface expression

These RAMP3 mutants generated were not tagged, thus the AM₂ receptor expression was estimated by measuring HA-CLR expression at the cell surface (Figure 5.1). The cell surface expression of CLR in complex with the RAMP3 mutants is shown in Figure 5.1. Compared to CLR alone, the cotransfection of RAMP3 induced a significant increase in CLR cell-surface expression ($p < 0.001$ by one-way ANOVA followed by Dunnett’s test). Apart from D46V which induced a small reduction in receptor expression at the cell surface, all the other seven mutant AM₂ receptors showed equivalent expression level to the WT AM₂ receptor (analysed by one-way ANOVA, followed by Dunnett’s test).

![Figure 5.1](image-url)

**Figure 5.1** ELISA data for the RAMP3 mutants expressed with HA-CLR, measuring HA-CLR expression at the cell surface. Data are mean ± s.e.m. of four independent experiments, each performed with eight replicates. *$p < 0.05$ vs WT by one-way ANOVA followed by Dunnett’s test.
5.2.1.2 Functional characterisation using cAMP assays

As demonstrated in Chapter 4, the CGRP receptor and AM$_2$ receptor phenotypes can be pharmacologically differentiated by their differential peptide selectivity. hαCGRP is ~1300-fold more potent at the CGRP receptor than at the AM$_2$ receptor whereas hAM is ~20-fold more potent at the AM$_2$ receptor than at the CGRP receptor. Therefore, hαCGRP and hAM responses were determined at the AM$_2$ receptors containing RAMP3 mutants. It was expected that hAM potency would be reduced whereas hαCGRP potency would be enhanced at these mutant AM$_2$ receptors, if the residues of RAMP3 replaced were important for the peptide to receptor interactions.

5.2.1.2.1 E35A AM$_2$ receptor

There was no significant difference in peptide response between the mutant E35A AM$_2$ receptor and the WT receptor for either hAM or hαCGRP (Figure 5.2). pEC$_{50}$ for hAM response at the mutant receptor was 9.92 ± 0.11 compared to 9.92 ± 0.15 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The E$_{\text{max}}$ values were 70.1 ± 4.91 for the mutant receptor and 72.4 ± 2.89 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.; n=3). hαCGRP responses were: pEC$_{50}$ ± s.e.m., mutant 7.27 ± 0.11 vs WT 7.50 ± 0.15 and E$_{\text{max}}$ ± s.e.m., mutant 84.0 ± 3.23 vs WT 85.4 ± 3.76 (n=4).

**Figure 5.2** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant E35A AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of three to four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.1.2.2 D46V AM$_2$ receptor

There was no significant difference in peptide response between the mutant D46V AM$_2$ receptor and the WT receptor for either hαCGRP or hAM (Figure 5.3). pEC$_{50}$ for hAM at the mutant receptor was 9.91 ± 0.11 compared to 9.92 ± 0.15 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The E$_{max}$ values were 66.4 ± 4.81 for the mutant receptor and 72.4 ± 2.89 for the WT receptor, respectively (E$_{max}$ ± s.e.m.; n=3). hαCGRP responses were: pEC$_{50}$ ± s.e.m., mutant 7.40 ± 0.08 vs WT 7.50 ± 0.15 and E$_{max}$ ± s.e.m., mutant 84.6 ± 0.68 vs WT 83.5 ± 2.00 (n=4).

Figure 5.3 cAMP data for (a) hAM and (b) hαCGRP responses at the mutant D46V AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of three to four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.1.2.3 E74W AM<sub>2</sub> receptor

hAM potency was significantly reduced at the mutant E74W AM<sub>2</sub> receptor (Figure 5.4a). There was a ~8-fold reduction in pEC<sub>50</sub> (pEC<sub>50</sub> ± s.e.m., mutant 9.21 ± 0.14 vs WT 10.1 ± 0.14, \( p < 0.01 \) by unpaired t-test, n=5) whereas the maximum response was not significantly altered (E<sub>max</sub> ± s.e.m., mutant 65.3 ± 4.86 vs WT 66.5 ± 2.86). On the other hand, there was no significant difference in peptide response between the mutant E74W AM<sub>2</sub> receptor and the WT receptor (Figure 5.4b). pEC<sub>50</sub> for hαCGRP at the mutant receptor was 7.08 ± 0.06 compared to 6.85 ± 0.11 for the WT receptor (pEC<sub>50</sub> ± s.e.m.; n=4). The E<sub>max</sub> values were 66.3 ± 3.27 for the mutant receptor and 68.9 ± 2.62 for the WT receptor, respectively (E<sub>max</sub> ± s.e.m.).

**Figure 5.4** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant E74W AM<sub>2</sub> receptor and the WT AM<sub>2</sub> receptor. The graphs are representative of five independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.1.2.4 P87A AM\textsubscript{2} receptor

There was no significant difference in peptide response between the mutant P87A AM\textsubscript{2} receptor and the WT receptor for either hAM or hαCGRP (Figure 5.5). pEC\textsubscript{50} for hAM at the mutant receptor was 10.2 ± 0.09 compared to 9.92 ± 0.13 at the WT receptor (pEC\textsubscript{50} ± s.e.m.; n=3). The E\textsubscript{max} values were 75.8 ± 4.87 for the mutant receptor and 70.3 ± 5.69 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m.). hαCGRP responses were: pEC\textsubscript{50} ± s.e.m., mutant 7.41 ± 0.03 vs WT 7.44 ± 0.13 and E\textsubscript{max} ± s.e.m., mutant 84.1 ± 2.51 vs WT 84.9 ± 3.64 (n=4).

**Figure 5.5** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant P87A AM\textsubscript{2} receptor and the WT AM\textsubscript{2} receptor. The graphs are representative of three to four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.1.2.5 L88E AM$_2$ receptor

There was no significant difference in peptide response between the mutant L88E AM$_2$ receptor and the WT receptor for either hAM or hαCGRP (Figure 5.6). pEC$_{50}$ for hAM at the mutant receptor was 10.0 ± 0.06 compared to 9.96 ± 0.10 for the WT receptor (pEC$_{50}$ ± s.e.m.; n=4). The E$_{\text{max}}$ values were 66.2 ± 5.38 for the mutant receptor and 67.8 ± 4.73 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). hαCGRP responses were: pEC$_{50}$ ± s.e.m., mutant 7.34 ± 0.07 vs WT 7.47 ± 0.14 and E$_{\text{max}}$ ± s.e.m., mutant 80.2 ± 3.17 vs WT 83.2 ± 2.51 (n=4).

![Figure 5.6](image)

**Figure 5.6** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant L88E AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.1.2.6 A89V AM2 receptor

hAM potency was significantly enhanced at the mutant A89V AM2 receptor (Figure 5.7a). There was a ~4-fold increase in potency (pEC$_{50}$ ± s.e.m., mutant 10.3± 0.13 vs WT 9.71 ± 0.15, $p < 0.05$ by unpaired t-test, n=3) whereas the maximum response was not significantly altered (E$_{\text{max}}$ ± s.e.m., mutant 71.2 ± 8.79 vs WT 63.9 ± 10.2, n=3). On the other hand, there was no significant difference in hαCGRP response between the mutant A89V AM2 receptor and the WT receptor (Figure 5.7b). pEC$_{50}$ for hαCGRP at the mutant receptor was 7.70 ± 0.03 compared to 7.39 ± 0.15 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=4). The E$_{\text{max}}$ values were 78.1 ± 2.04 for the mutant receptor and 83.3 ± 2.49 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.).

**Figure 5.7** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant A89V AM2 receptor and the WT AM2 receptor. The graphs are representative of three to four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.1.2.7 I93F AM$_2$ receptor

There was no significant difference in peptide response between the mutant I93F AM$_2$ receptor and the WT receptor for either hAM or hαCGRP (Figure 5.8). pEC$_{50}$ for hAM at the mutant receptor was 9.93 ± 0.04 compared to 9.81 ± 0.14 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=4). The E$_{\text{max}}$ values were 62.6 ± 9.78 for the mutant receptor and 66.8 ± 7.64 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). hαCGRP responses were: pEC$_{50}$ ± s.e.m., mutant 7.10 ± 0.14 vs WT 7.36 ± 0.14 and E$_{\text{max}}$ ± s.e.m., mutant 81.1 ± 2.55 vs WT 83.5 ± 2.81 (n=4).

![Figure 5.8](image-url)

**Figure 5.8** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant I93F AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.1.2.8 N103S AM$_2$ receptor

There was no significant difference in peptide response between the mutant N103S AM$_2$ receptor and the WT receptor for either hAM or hαCGRP (Figure 5.9). pEC$_{50}$ for hAM at the mutant receptor was 10.1 ± 0.09 compared to 9.99 ± 0.18 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The E$_{\text{max}}$ values were 68.9 ± 6.45 for the mutant receptor and 73.6 ± 3.79 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). hαCGRP responses were: pEC$_{50}$ ± s.e.m., mutant 7.27 ± 0.05 vs WT 7.32 ± 0.16 and E$_{\text{max}}$ ± s.e.m., mutant 79.5 ± 1.89 vs WT 81.4 ± 3.53 (n=4).

**Figure 5.9** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant N103S AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of three to four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.1.3 Characterisation of RAMP1_{86-89} AM\_2 receptor

Data generated from the RAMP1 mutant, RAMP3_{86-89}, with CLR suggested that the region in RAMP1 might be important for hAM and hαCGRP, but not hβCGRP interactions with the receptor. To elucidate if the corresponding region in RAMP3 plays a similar role, the reciprocal RAMP3 mutant (RAMP1_{86-89}) containing the triple mutations P87A, L88E and A89V were generated and characterised with CLR for both receptor expression and function.

Cell-surface expression of the mutant and WT AM\_2 receptors were estimated by measuring HA-CLR expression in ELISA; the mutant RAMP1_{86-89} AM\_2 receptor showed comparable expression to the WT AM\_2 receptor at the cell surface (Figure 5.10a). Furthermore, there was no significant difference in any of hαCGRP, hAM or hβCGRP response between the mutant RAMP1_{86-89} AM\_2 receptor and WT AM\_2 receptor (Figure 5.10b-d). The pEC\_50 values were pEC\_50 \pm s.e.m.; hαCGRP, mutant 6.85 \pm 0.12, WT 6.81 \pm 0.15 (n=3); hAM, mutant 9.75 \pm 0.13, WT 9.73 \pm 0.12 (n=4); hβCGRP, mutant 7.93 \pm 0.14, WT 7.61 \pm 0.25 (n=3). The E\_max values were E\_max \pm s.e.m.; hαCGRP, mutant 62.8 \pm 5.56, WT 58.1 \pm 5.72; hAM, mutant 76.2 \pm 3.91, WT 73.4 \pm 4.10; hβCGRP, mutant 32.3 \pm 7.27, WT 60.3 \pm 6.15.
Figure 5.10 (a) ELISA data for RAMP1_{86-89} expressed with HA-CLR, measuring HA-CLR expression at the cell surface. Data are mean ± s.e.m. of three independent experiments, each performed with eight replicates. (b) cAMP data for hαCGRP, (c) hAM and (d) hβCGRP responses at RAMP1_{86-89} AM₂ and WT AM₂. The graphs are representative of 3 independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.2 RAMP3 mutants with HA-\textit{CT}_{(a)}; AMY_{3(a)} receptor

The effects of the eight RAMP3 mutants were also studied in the AMY_{3(a)} receptor by coexpressing with CT_{(a)}. In this part of study, it was hypothesised that these residues in RAMP3 might play a role in defining AMY_{3(a)} receptor specific pharmacology. Unlike RAMP1 mutants which are tagged, the RAMP3 mutants generated in this chapter were not tagged. In addition, CT_{(a)} alone has significant expression at the cell surface, thus it is technically not possible to directly measure the RAMP3/HA-\textit{CT}_{(a)} complex expression. Expression data generated from the RAMP3 mutants with HA-CLR can provide some indication for the ability of the RAMP3 mutants in associating with a GPCR (Figure 5.1). Nevertheless, the use of these data may be limited and need to be carefully interpreted. The RAMP1 to CLR association seems to be different from its association with CT_{(a)}; the expression profiles for the RAMP1 mutants with CLR and CT_{(a)} appeared to be very different (Chapter 4).

In cAMP assays, the responses of two peptide agonists, rAmy and hβCGRP, were determined. As demonstrated in Chapter 4, rAmy is equally potent at AMY_{1(a)} and AMY_{3(a)} whereas hβCGRP is more potent at AMY_{1(a)} than at AMY_{3(a)}. Therefore, if the residues were important for the peptide to receptor interactions, it was expected to see an enhanced hβCGRP potency but an unaffected rAmy potency at the AMY_{3(a)} receptors containing these RAMP3 mutants.
5.2.2.1 E35A AMY3(a) receptor

There was no significant difference in peptide response between the mutant E35A AMY3(a) receptor and the WT receptor for either rAmy or hβCGRP (Figure 5.11). pEC$_{50}$ for rAmy response at the mutant receptor was 9.85 ± 0.25 compared to 10.3 ± 0.16 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The E$_{\text{max}}$ values were 77.2 ± 5.02 for the mutant receptor and 83.6 ± 1.63 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). hβCGRP responses were: pEC$_{50}$ ± s.e.m., mutant 9.16 ± 0.28 vs WT 9.36 ± 0.14 and E$_{\text{max}}$ ± s.e.m., mutant 74.5 ± 6.57 vs WT 89.3 ± 5.77 (n=4).

![Graph](image)

**Figure 5.11** cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant E35A AMY3(a) receptor and the WT AMY3(a) receptor. The graphs are representative of three to four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.2.2 D46V AMY3(a) receptor

There was no significant difference in peptide response between the mutant D46V AMY3(a) receptor and the WT receptor for either rAmy or hβCGRP (Figure 5.12). pEC\(_{50}\) for rAmy response at the mutant receptor was 10.3 ± 0.22 compared to 10.3 ± 0.16 at the WT receptor (pEC\(_{50}\) ± s.e.m.; n=3). The E\(_{\text{max}}\) values were 73.7 ± 6.75 for the mutant receptor and 85.8 ± 3.38 for the WT receptor, respectively (E\(_{\text{max}}\) ± s.e.m.). hβCGRP responses were: pEC\(_{50}\) ± s.e.m., mutant 9.37 ± 0.23 vs WT 9.36± 0.14 and E\(_{\text{max}}\) ± s.e.m., mutant 79.5 ± 5.51 vs WT 89.3 ± 5.77 (n=4).

**Figure 5.12** cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant D46V AMY3(a) receptor and the WT AMY3(a) receptor. The graphs are representative of three to four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.2.3 E74W AMY<sub>3(a)</sub> receptor

Both rAmy and hβCGRP potencies were significantly reduced at the mutant E74W AMY<sub>3(a)</sub> receptor compared to the WT receptor (Figure 5.13). pEC<sub>50</sub> for rAmy response at the mutant receptor was 9.87 ± 0.09 compared to 10.7 ± 0.08 at the WT receptor (pEC<sub>50</sub> ± s.e.m., p < 0.001 by unpaired t-test, n=4), displaying a ~7-fold reduction. The E<sub>max</sub> values were not significantly affected as 76.8 ± 5.54 for the mutant receptor and 88.1 ± 2.13 for the WT receptor, respectively (E<sub>max</sub> ± s.e.m.). There was a ~3-fold reduction in hβCGRP potency (pEC<sub>50</sub> ± s.e.m., mutant 9.60 ± 0.14 vs WT 10.1± 0.11, p < 0.05 by unpaired t-test, n=3) whereas maximum response was not significantly altered (E<sub>max</sub> ± s.e.m., mutant 81.8 ± 2.60 vs WT 85.1 ± 1.27, n=3).

Figure 5.13 cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant E74W AMY<sub>3(a)</sub> receptor and the WT AMY<sub>3(a)</sub> receptor. The graphs are representative of three to four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.2.4 P87A AMY₃(a) receptor

There was no significant difference in peptide response between the mutant P87A AMY₃(a) receptor and the WT receptor for either rAmy or hβCGRP (Figure 5.14). pEC₅₀ for rAmy response at the mutant receptor was 10.2 ± 0.12 compared to 10.6 ± 0.14 at the WT receptor (pEC₅₀ ± s.e.m.; n=4). The Eₘₐₓ values were 79.2 ± 8.41 for the mutant receptor and 93.0 ± 4.41 for the WT receptor, respectively (Eₘₐₓ ± s.e.m.). hβCGRP responses were: pEC₅₀ ± s.e.m., mutant 9.83 ± 0.21 vs WT 10.1± 0.11 and Eₘₐₓ ± s.e.m., mutant 80.4 ± 4.92 vs WT 85.6 ± 0.88 (n=3).

![Figure 5.14 cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant P87A AMY₃(a) receptor and the WT AMY₃(a) receptor. The graphs are representative of three to four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.](image-url)
5.2.2.5 L88E AMY$_{3(a)}$ receptor

There was no significant difference in peptide response between the mutant L88E AMY$_{3(a)}$ receptor and the WT receptor for rAmy (Figure 5.15a). pEC$_{50}$ for rAmy response at the mutant receptor was 10.2 ± 0.20 compared to 10.7 ± 0.13 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The E$_{\text{max}}$ values were 84.6 ± 6.76 for the mutant receptor and 95.2 ± 5.44 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.). hβCGRP potency was not significantly affected by the L88E mutation (pEC$_{50}$ ± s.e.m., mutant 9.73 ± 0.12 vs WT 10.1 ± 0.10, n=3), whilst there was a very small but significant reduction in maximum response (E$_{\text{max}}$ ± s.e.m., mutant 79.4 ± 1.93 vs WT 86.0 ± 1.32, $p < 0.05$, n=3) (Figure 5.15b).

![Figure 5.15](image)

**Figure 5.15** cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant L88E AMY$_{3(a)}$ receptor and the WT AMY$_{3(a)}$ receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.2.6 A89V AMY_3(a) receptor

There was no significant difference in peptide response between the mutant A87V AMY_3(a) receptor and the WT receptor for either rAmy or hβCGRP (Figure 5.16). pEC_{50} for rAmy response at the mutant receptor was 10.5 ± 0.02 compared to 10.7 ± 0.13 at the WT receptor (pEC_{50} ± s.e.m.; n=3). The E_{max} values were 86.1 ± 6.78 for the mutant receptor and 95.2 ± 5.44 for the WT receptor, respectively (E_{max} ± s.e.m.). hβCGRP responses were: pEC_{50} ± s.e.m., mutant 10.2 ± 0.13 vs WT 10.0 ± 0.11 and E_{max} ± s.e.m., mutant 85.9 ± 4.68 vs WT 85.3 ± 1.73 (n=3).

Figure 5.16 cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant A89V AMY_3(a) receptor and the WT AMY_3(a) receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.2.7 I93F AMY$_{3(a)}$ receptor

Both rAmy and hβCGRP potencies were significantly reduced at the mutant I93F AMY$_{3(a)}$ receptor compared to the WT receptor (Figure 5.17). pEC$_{50}$ for rAmy response at the mutant receptor was 10.1 ± 0.07 compared to 10.7 ± 0.17 at the WT receptor (pEC$_{50}$ ± s.e.m., p < 0.05 by unpaired t-test, n=4), displaying a ~4-fold reduction. The E$_{max}$ values were not significantly affected as 84.6 ± 2.20 for the mutant receptor and 89.3 ± 1.77 for the WT receptor, respectively (E$_{max}$ ± s.e.m.). There was a ~7-fold reduction in hβCGRP potency (pEC$_{50}$ ± s.e.m., mutant 9.14 ± 0.13 vs WT 10.0± 0.07, p < 0.01 by unpaired t-test, n=4) whereas maximum response was not significantly altered (E$_{max}$ ± s.e.m., mutant 75.5 ± 3.38 vs WT 84.9 ± 2.02).

![Figure 5.17](image)

**Figure 5.17** cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant I93F AMY$_{3(a)}$ receptor and the WT AMY$_{3(a)}$ receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.2.2.8 N103S AMY<sub>3(a)</sub> receptor

Both rAmy and hβCGRP potencies were significantly reduced at the mutant receptor compared to the WT receptor (Figure 5.18). pEC<sub>50</sub> for rAmy response at the mutant receptor was 9.69 ± 0.08 compared to 10.8 ± 0.15 at the WT receptor (pEC<sub>50</sub> ± s.e.m., p < 0.01 by unpaired t-test, n=3), displaying a ~13-fold reduction. The E<sub>max</sub> values were not significantly affected as 80.2 ± 5.23 for the mutant receptor and 87.2 ± 1.16 for the WT receptor, respectively (E<sub>max</sub> ± s.e.m.). There was a a ~11-fold reduction in hβCGRP potency (pEC<sub>50</sub> ± s.e.m., mutant 9.06 ± 0.02 vs WT 10.1± 0.07, p < 0.001 by unpaired t-test, n=3) and maximum response was also significantly reduced (E<sub>max</sub> ± s.e.m., mutant 75.5 ± 1.72 vs WT 86.0 ± 2.88, p < 0.05 by unpaired t-test).

![Graph](image)

**Figure 5.18** cAMP data for (a) rAmy and (b) hβCGRP responses at the mutant N103S AMY<sub>3(a)</sub> receptor and the WT AMY<sub>3(a)</sub> receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
5.3 Discussion

Consistent with the previously published data (Hay et al., 2006a), E74W AM2 showed selectively reduced hAM potency, but hαCGRP response and receptor cell-surface expression was not affected. This reduced hAM potency appeared to be exactly opposite to the effect induced by the reciprocal RAMP1 mutant W74E (Chapter 4), suggesting the importance of E74 to hAM potency; therefore further characterisation was performed with both E74W RAMP3 and W74E RAMP1 in complex with CLR (Refer to Chapter 6). In addition, the data have emphasised that position 74 may not be important to hαCGRP to receptor interactions, which is consistent with other studies (Hay et al., 2006a; Qi et al., 2008). Besides E74W, the A89V RAMP3 mutant also displayed interesting behaviour with an increase in hAM potency in the absence of any change in CGRP potency or expression of the receptor complex. However, no change in hAM or hαCGRP potency was observed with its reciprocal RAMP1 mutant V89A in Chapter 4. It is possible that introduction of the branched side-chain of valine enabled interaction with other residues in close proximity in RAMP3 leading to enhanced hAM binding or a small conformational effect on the receptor complex. On the other hand, loss of this side-chain in RAMP1 is apparently not detrimental to AM/CGRP interactions at CGRP receptors.

The reciprocal RAMP3 mutant to RAMP3_{86-89} with the residues replaced by the corresponding RAMP1 residue in position 86-89 (RAMP1_{86-89}) was in addition analysed with CLR. Unlike RAMP3_{86-89} which caused significant reduction in hαCGRP and hAM potency, RAMP1_{86-89} failed to elicit any changes to peptide responses for hαCGRP, hAM and hβCGRP. The lack of effect in RAMP3 substitutions suggests that position 86-89 may have different role in ligand to receptor interactions; whilst region 86-89 in RAMP1 contributes selectively to hαCGRP and hAM but not hβCGRP interactions with the CGRP receptor, the same region in RAMP3 does not seem to have a role in ligand interactions with the AM2 receptor.

The eight RAMP3 point mutants were also characterised in the complexes with CT_{(a)}. Like RAMP1 mutants, peptide responses generated from coexpression of RAMP3 point mutants with CT_{(a)} showed more changes than with CLR. Parallel reductions in potency were observed for hβCGRP and rAmy at E74W RAMP3, I93F RAMP3 and N103S RAMP3. Although direct assessment of this was not possible as these RAMP3 mutants were not tagged, the generalised perturbations in function may most likely relate to altered receptor
expression at the cell surface. The expression data generated from the receptors containing the RAMP1 mutants in Chapter 4 has suggested that the expression of the receptor formed with CLR did not correlate well with that of the receptors formed with CT \(_{(a)}\). In addition, the RAMP1 point mutants which showed significant alterations to peptide potency in the AMY \(_{1(a)}\) receptors were all paralleled by altered cell-surface expression (Chapter 4).
Chapter 6

Structure-function relationships of residues at position 74 of RAMP1 and RAMP3 in CGRP and AM\textsubscript{2} receptors

6.1 Introduction

As demonstrated in Chapter 5, E74W selectively reduced hAM potency in AM\textsubscript{2} receptor but hαCGRP potency was unaffected. This observation was consistent with the previous study (Hay et al., 2006a). An exactly opposite effect was seen with the reciprocal RAMP1 mutant W74E in the CGRP receptor where hAM potency was enhanced similarly and CGRP potency was unchanged (Chapter 4). The reciprocal nature of the effects for hAM responses suggests that E74 of RAMP3 is important for AM interactions with the AM\textsubscript{2} receptor. Comparing the amino acid properties of the two native residues at position 74 in RAMPs 1 and 3, it reveals that W in RAMP1 has a bulky side chain consisting of aromatic groups whereas Glu in RAMP3 has a relatively small side chain which is negatively charged. Amino acid substitutions carried out in a previous study showed that E74K and E74Q also reduced hAM potency whilst E74D substitution showed hAM potency that was not significantly different from WT AM\textsubscript{2} receptor (Hay et al., 2006a). It is clear that the interaction at position 74 is facilitated by some amino acids but not others. Therefore, to provide greater insight into the role of the residue at position 74 of RAMPs 1 and 3, additional characterisations have been performed at this position in this chapter.

To further determine whether the residue at position 74 in RAMP3 is important for other form or related peptides of AM, two additional peptides, a peptide fragment of hAM that lacks the first 14 amino acids, hAM\textsubscript{15-52} and hAM\textsubscript{2} (47 amino acids) were assayed at both E74W AM\textsubscript{2} and W74E CGRP receptors. In addition, whilst data generated in the functional assay has suggested that E74 is important for AM potency, it is not yet known whether the altered AM potency was a result of the change in the AM binding to the receptor. Therefore, the binding profiles for \textsuperscript{125}I-AM\textsubscript{13-52} were also determined at these two mutant receptors and their corresponding WT receptors in this chapter.

In addition, further amino acid substitutions were carried out at position 74 in RAMPs 1 and 3 in the chapter, aiming to define the amino acid properties that are required for the interaction at position 74 of RAMP 3. Eight amino acids representing a broad spectrum of chemical properties were selected and individually substituted into position 74 in RAMP3 to
replace the native Glu. These were: Trp (bulky, containing an aromatic side chain), Phe (bulky, containing an aromatic side chain), Tyr (bulky, containing an aromatic side chain), Ala (small, containing no side chain), Ser (small, containing a hydroxyl group), Thr (small, containing a hydroxyl group), Arg (positively charged) and Asn (polar, containing an amide group). Five amino acid substitutions (Glu, Phe, Tyr, Ala and Asn) were also made in RAMP1 at position 74 to replace the native Trp. Though E74W RAMP3 and W74E substitutions have been characterised with CLR in Chapter 5 and 4, they were included for analysis again to allow direct comparison with the other substitutions characterised in this chapter. Similar to the characterisations performed in Chapters 4 and 5, these RAMP1 or RAMP3 mutants were analysed with HA-CLR and peptide responses for hAM and hαCGRP were determined.
6.2 Results

6.2.1 Additional characterisation at E74W AM$_2$ and W74E CGRP receptors

6.2.1.1 hAM2

The hAM2 potency was significantly altered at both E74W AM$_2$ and W74E CGRP receptors (Figure 6.1). pEC$_{50}$ for the E74W AM$_2$ receptor was 9.85 ± 0.09 compared to 10.14 ± 0.02 at the WT receptor (pEC$_{50}$ ± s.e.m., $p < 0.05$ by unpaired t test, n=4), displaying a ~2-fold reduction in potency. The maximum response was also significantly reduced at the mutant receptor (E$_{\text{max}}$ ± s.e.m., mutant 67.8 ± 2.62 vs WT 78.4 ± 1.47, $p < 0.05$ by unpaired t test). In contrast, there was a ~5-fold increase in hAM2 potency at W74E CGRP receptor (pEC$_{50}$ ± s.e.m., mutant 9.76 ± 0.06 vs WT 9.07 ± 0.07, $p < 0.001$ by unpaired t test, n=4). The maximum response was also significantly enhanced (E$_{\text{max}}$ ± s.e.m., mutant 78.5 ± 2.83 vs WT 67.8 ± 0.86, $p < 0.05$ by unpaired t test).

\[ \log[hAM2] \]
\[ \text{cAMP % forskolin} \]
\[ 0 \quad -12 
-11 
-10 
-9 
-8 
-7 
-6 \]

Figure 6.1 cAMP data for hAM2 response at (a) E74W AM$_2$ receptor and (b) W74E CGRP receptor with the WT receptors. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.1.2 hAM<sub>15-52</sub>

hAM<sub>15-52</sub> potency was also significantly altered at both E74W AM<sub>2</sub> and W74E CGRP receptors but the maximum response was unaffected (Figure 6.2). pEC<sub>50</sub> for E74W AM<sub>2</sub> receptor was 8.84 ± 0.04 compared to 9.69 ± 0.08 at the WT receptor (pEC<sub>50</sub> ± s.e.m., p < 0.001 by unpaired t test, n=4), displaying a ~7-fold reduction. The maximum response was not significantly affected; 69.6 ± 4.32 for E74W AM<sub>2</sub> and 77.7 ± 1.46 for the WT receptor, respectively (E<sub>max</sub> ± s.e.m.). In contrast, there was a ~10-fold increase in hAM<sub>15-52</sub> potency at the W74E CGRP receptor (pEC<sub>50</sub> ± s.e.m., mutant 9.14 ± 0.05 vs WT 8.14 ± 0.08, p < 0.001 by unpaired t test, n=4) whereas the maximum response was not significantly altered (E<sub>max</sub> ± s.e.m., mutant 80.2 ± 3.28 vs WT 75.5 ± 6.62).

![Graph](image)

**Figure 6.2** cAMP data for hAM<sub>15-52</sub> response at (a) E74W AM<sub>2</sub> receptor and (b) W74E CGRP receptor with the WT receptors. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.1.3 $^{125}$I-hAM$_{13-52}$ binding

The binding profile of $^{125}$I-hAM$_{13-52}$ was determined at the E74W AM$_2$ and W74E CGRP receptors. Peptide fragment 13-52 of hAM was used instead the full length hAM due to its commercial availability. It has been demonstrated previously that $^{125}$I-hAM$_{13-52}$ is a better radioligand than $^{125}$I-hAM$_{1-52}$ for binding characterisations as it offers better specificity and affinity (Juaneda et al., 2003). In addition, data generated from hAM$_{15-52}$ response at these two mutant receptors have eliminated a role of the first 14 amino acid in the hAM interactions to position 74 of RAMP3. Thus, it was hypothesised that E74 of RAMP3 had a role in hAM binding to the AM$_2$ receptor, either directly or indirectly and the residues involved lie beyond residue 14 in hAM. This hypothesis was in addition supported by the “two-domain” binding mechanism for the family B GPCRs (Hoare, 2005) where the C-terminal domain of the peptide binds to the N-terminal domain of the receptor in “step 1”. Therefore, if E74 of RAMP3 was involved in hAM binding to the receptor, a similar reciprocal effect to that seen in hAM potency would also be observed in the $^{125}$I-hAM$_{13-52}$ binding at the two mutant receptors.

$^{125}$I-hAM$_{13-52}$ binding was significantly altered at both E74W AM$_2$ and W74E CGRP receptors. The specific binding of $^{125}$I-hAM$_{13-52}$ was significantly reduced at E74W AM$_2$ compared with WT (Figure 6.3c); this was accompanied by a ~14-fold reduction in AM affinity (Figure 6.3a) (pIC$_{50}$ ± s.e.m., mutant 7.42 ± 0.12 vs WT 8.58 ± 0.09, p < 0.01 by unpaired t test, n=3). In contrast, there was greater binding of $^{125}$I-hAM$_{13-52}$ at the W74E CGRP mutant than WT (Figure 6.3d), with a ~3-fold increase in AM affinity (pIC$_{50}$ ± s.e.m., mutant 7.73 ± 0.24 vs WT 7.22 ± 0.28, p < 0.01 by unpaired t-test, n=5).
Figure 6.3 Binding of $^{125}$I-hAM$_{13-52}$ to membrane preparations from cells transfected with WT or (a) mutant E74W AM$_2$ or (b) W74E CGRP receptors. The graphs are representative of three (a) or five (b) independent experiments. Data points are mean ± s.e.m. of duplicate assay points. (c) and (d) show the combined mean maximum specific binding of the WT or mutant receptors for E74W AM$_2$ or W74E CGRP receptors, respectively. *$p < 0.05$ (c) or **$p < 0.01$ (d) vs WT by unpaired t-test.
6.2.2 Amino acid substitutions at position 74 in RAMPs 1 and 3

6.2.2.1 RAMP3 mutants with CLR; $\text{AM}_2$ receptor

6.2.2.1.1 Cell-surface expression

The cell-surface expression of $\text{AM}_2$ receptors formed by the RAMP3 mutants and HA-CLR was estimated by measuring the HA-CLR expression in whole-cell ELISA (Figure 6.4). Compared to CLR alone, the cotransfection of RAMP3 induced a significant increase in CLR cell-surface expression ($p < 0.001$ by one-way ANOVA followed by Dunnett’s test). All the mutant $\text{AM}_2$ receptors had equivalent expression level at the cell surface to the WT $\text{AM}_2$ receptor.

![Figure 6.4 ELISA data for RAMP3 mutants expressed with HA-CLR, measuring HA-CLR expression at the cell surface. Data are mean ± s.e.m. of three independent experiments, each performed with eight replicates. All data were compared with WT by one-way ANOVA followed by Dunnett’s test, significance were achieved at $p < 0.05$.](image)
6.2.2.1.2 cAMP assay

6.2.2.1.2.1 E74W AM$_2$ receptor

The hAM potency was significantly reduced at the mutant E74W AM$_2$ receptor compared to the WT receptor (Figure 6.5a). There was a ~6-fold reduction in pEC$_{50}$ value (pEC$_{50}$ ± s.e.m., mutant 8.93 ± 0.12 vs WT 9.74 ± 0.13, $p < 0.05$ by paired t-test, n=4) whereas the maximum response was not significantly altered (E$_{\text{max}}$ ± s.e.m., mutant 73.6 ± 5.81 vs WT 78.7 ± 6.40). On the other hand, hαCGRP potency was slightly enhanced by ~2-fold at the mutant E74W AM$_2$ receptor (Figure 6.5b). pEC$_{50}$ for hαCGRP at the mutant AM$_2$ receptor was 7.08 ± 0.06 compared to 6.85 ± 0.11 for the WT receptor (pEC$_{50}$ ± s.e.m., $p < 0.05$ by paired t-test, n=4). The E$_{\text{max}}$ values were 66.3 ± 3.27 for the mutant receptor and 68.9 ± 2.62 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.).

Figure 6.5 cAMP data for (a) hAM and (b) hαCGRP responses at the mutant E74W AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.2.1.2.2 E74F AM$_2$ receptor

The hAM potency was significantly reduced at the mutant E74F AM$_2$ receptor compared to the WT receptor (Figure 6.6a). There was a ~4-fold reduction in hAM potency (pEC$_{50}$ ± s.e.m., mutant 9.16 ± 0.16 vs WT 9.76 ± 0.18, $p < 0.05$ by paired t-test, n=4) whereas maximum response was not significantly altered; E$_{\text{max}}$ ± s.e.m., mutant 76.2 ± 2.96 vs WT 80.4 ± 4.62. On the other hand, hαCGRP potency was slightly enhanced by ~2-fold at the mutant E74F AM$_2$ receptor (Figure 6.6b). pEC$_{50}$ for hαCGRP at the mutant receptor was 7.00 ± 0.17 compared to 6.77 ± 0.17 for the WT receptor (pEC$_{50}$ ± s.e.m., $p < 0.01$ by paired t-test, n=4). The E$_{\text{max}}$ value was not significantly affected as 66.2 ± 5.02 for the mutant receptor and 69.9 ± 3.51 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.).

![Graphs showing cAMP data for hAM and hαCGRP responses at the mutant E74F AM$_2$ receptor and the WT AM$_2$ receptor.](Image)

**Figure 6.6** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant E74F AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.2.1.2.3 E74YAM$_2$ receptor

There was no significant difference in peptide response between the mutant E74Y AM$_2$ receptor and the WT receptors for either hAM or hαCGRP (Figure 6.7). pEC$_{50}$ for hAM at the mutant receptor $9.38 \pm 0.08$ compared to $9.73 \pm 0.17$ at the WT receptor (pEC$_{50}$ \pm s.e.m.; n=4). $E_{\text{max}}$ values were $81.1 \pm 4.73$ for the mutant receptor and $79.8 \pm 4.73$ for the WT receptor, respectively ($E_{\text{max}} \pm \text{s.e.m.}$). hαCGRP responses were: pEC$_{50}$ \pm s.e.m., mutant $6.94 \pm 0.10$ vs WT $6.78 \pm 0.16$ and $E_{\text{max}} \pm \text{s.e.m.}$, mutant $75.2 \pm 0.79$ vs WT $75.1 \pm 2.21$ (n=4).

![Cyclic AMP (cAMP) data for (a) hAM and (b) hαCGRP responses at the mutant E74Y AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of four independent experiments. Data points are mean \pm s.e.m. of triplicate assay points.](image)

Figure 6.7 cAMP data for (a) hAM and (b) hαCGRP responses at the mutant E74Y AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of four independent experiments. Data points are mean \pm s.e.m. of triplicate assay points.
6.2.2.1.2.4 E74A AM\textsubscript{2} receptor

The hAM potency was significantly reduced at the mutant E74A AM\textsubscript{2} receptor compared to the WT receptor (Figure 6.8a). There was a ~3-fold reduction in pEC\textsubscript{50} value (pEC\textsubscript{50} ± s.e.m., mutant 9.30 ± 0.13 vs WT 9.77 ± 0.14, \( p < 0.05 \) by paired t-test, \( n=4 \)) and the maximum response was not significantly different (E\textsubscript{max} ± s.e.m., mutant 78.0 ± 6.18 vs WT 80.2 ± 4.58).

On the other hand, there was no significant difference in hαCGRP potency between the mutant E74A AM\textsubscript{2} and the WT receptor (Figure 6.8b) pEC\textsubscript{50} for hαCGRP at the mutant receptor was 6.99 ± 0.06 compared to 6.86 ± 0.08 for the WT receptor (pEC\textsubscript{50} ± s.e.m.; \( n=4 \)). The E\textsubscript{max} was slightly reduced; 70.4 ± 0.70 for the mutant receptor and 76.9 ± 1.01 for the WT receptor, respectively (E\textsubscript{max} ± s.e.m, \( p < 0.05 \) by paired t-test).

**Figure 6.8** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant E74A AM\textsubscript{2} receptor and the WT AM\textsubscript{2} receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.1.2.5 E74S AM$_2$ receptor

The hAM potency was significantly reduced at the mutant E74S AM$_2$ receptor (Figure 6.9a). There was a ~3-fold reduction in pEC$_{50}$ value (pEC$_{50}$ ± s.e.m., mutant 9.21 ± 0.07 vs WT 9.66 ± 0.06, $p < 0.001$ by paired t-test, n=4) whereas the maximum response was not significantly altered (E$_{\text{max}}$ ± s.e.m., mutant 68.6 ± 5.03 vs WT 68.4 ± 4.87). On the other hand, there was no significant difference in hαCGRP response between the mutant E74S AM$_2$ and the WT receptor (Figure 6.9b) pEC$_{50}$ for hαCGRP at the mutant receptor was 6.87 ± 0.15 compared to 6.68 ± 0.22 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=4). The E$_{\text{max}}$ values were 63.1 ± 6.13 for the mutant receptor and 60.4 ± 5.95 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.).

![Graphs](image)

**Figure 6.9** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant E74S AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.1.2.6 E74T AM2 receptor

The hAM potency was significantly reduced at the mutant E74T AM2 receptor (Figure 6.10a). There was a ~2-fold reduction in pEC50 value (pEC50 ± s.e.m., mutant 9.32 ± 0.06 vs WT 9.66 ± 0.06, p < 0.05 by paired t-test, n=4) whereas the maximum response was not significantly altered (E_{max} ± s.e.m., mutant 67.1 ± 6.07 vs WT 68.4 ± 4.78). On the other hand, there was no significant difference in hαCGRP response between the mutant E74T AM2 and the WT receptor (Figure 6.10b). pEC50 for hαCGRP at the mutant receptor was 6.85 ± 0.05 compared to 6.68 ± 0.22 at the WT receptor (pEC50 ± s.e.m.; n=4). The E_{max} values were 60.5 ± 6.78 for the mutant receptor and 60.4 ± 5.95 for the WT receptor, respectively (E_{max} ± s.e.m.).

**Figure 6.10** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant E74T AM2 receptor and the WT AM2 receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.2.1.2.7 E74R AM$_2$ receptor

The hAM potency was significantly reduced at the mutant E74R AM$_2$ receptor (Figure 6.11a). There was a ~3-fold reduction in pEC$_{50}$ value (pEC$_{50}$ ± s.e.m., mutant 9.10 ± 0.18 vs WT 9.55 ± 0.17, $p < 0.001$ by paired t-test, n=4) whereas the maximum response was not significantly altered ($E_{\text{max}}$ ± s.e.m., mutant 63.4 ± 4.05 vs WT 62.8 ± 2.45). On the other hand, there was no significant difference in hαCGRP response between the mutant E74R AM$_2$ and the WT receptor (Figure 6.11a) pEC$_{50}$ for hαCGRP at the mutant receptor was 7.05 ± 0.01 compared to 6.85 ± 0.10 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The $E_{\text{max}}$ values were 62.2 ± 1.94 for the mutant receptor and 66.6 ± 3.28 for the WT receptor, respectively ($E_{\text{max}}$ ± s.e.m.).

Figure 6.11 cAMP data for (a) hAM and (b) hαCGRP responses at the mutant E74R AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of three to four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.2.1.2.8 E74N AM$_2$ receptor

The hAM potency was significantly reduced at the mutant E74N AM$_2$ receptor (Figure 6.12a). There was a ~4-fold reduction in pEC$_{50}$ value (pEC$_{50}$ ± s.e.m., mutant 9.19 ± 0.08 vs WT 9.74 ± 0.17, $p < 0.05$ by paired t-test, n=4) whereas the maximum response was not significantly altered (E$_{\text{max}}$ ± s.e.m., mutant 75.7 ± 3.51 vs WT 79.9 ± 4.76). On the other hand, there was no significant difference in hαCGRP response between the mutant E74N AM$_2$ and the WT receptor for hαCGRP response (Figure 6.12b). pEC$_{50}$ for hαCGRP at the mutant receptor was 6.76 ± 0.14 compared to 6.78 ± 0.16 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=4). The E$_{\text{max}}$ values were 66.9 ± 3.99 for the mutant receptor and 75.1 ± 2.21 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.).

Figure 6.12 cAMP data for (a) hAM and (b) hαCGRP responses at the mutant E74N AM$_2$ receptor and the WT AM$_2$ receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.2.2 RAMP1 mutants with HA-CLR; CGRP receptor

6.2.2.2.1 Cell-surface expression

The cell-surface expression of CGRP receptors formed by the RAMP1 mutants and HA-CLR was estimated by measuring the HA-CLR expression in whole-cell ELISA (Figure 6.13). Compared to CLR alone, the cotransfection of RAMP1 induced a significant increase in CLR cell-surface expression \( p < 0.001 \) by one-way ANOVA followed by Dunnett’s test. All mutant CGRP receptors had equivalent expression level at the cell surface to the WT CGRP receptor as estimated by HA-CLR expression.

![Figure 6.13](image)

**Figure 6.13** ELISA data for mycRAMP1 mutants expressed with HA-CLR, measuring HA-CLR expression at the cell surface. Data are mean ± s.e.m. of three independent experiments, each performed with four replicates. Data were analysed by one-way ANOVA, followed by Dunnett’s test. Significance was achieved at \( p < 0.05 \).

6.2.2.2.2 Functional characterisation using cAMP assays
6.2.2.2.2.1 W74E CGRP receptor

The hAM potency was significantly enhanced at the mutant W74E CGRP receptor (Figure 6.14a). There was a ~8-fold increase in pEC$_{50}$ value (pEC$_{50}$ ± s.e.m., mutant 8.95 ± 0.20 vs WT 8.04 ± 0.24, $p < 0.05$ by paired t-test, n=3) whereas the maximum response was not significantly altered ($E_{\text{max}}$ ± s.e.m., mutant 74.7 ± 3.76 vs WT 73.9 ± 4.23). On the other hand, there was no significant difference in hαCGRP response between the mutant W74E CGRP and the WT receptor (Figure 6.14b) pEC$_{50}$ for hαCGRP at the mutant receptor was 9.90 ± 0.17 compared to 9.88 ± 0.13 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The $E_{\text{max}}$ values were 77.8 ± 6.34 for the mutant receptor and 76.5 ± 3.17 for the WT receptor, respectively ($E_{\text{max}}$ ± s.e.m.).

Figure 6.14 cAMP data for (a) hAM and (b) hαCGRP responses at the mutant W74E CGRP receptor and the WT CGRP receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.2.2.2 W74F CGRP receptor

There was no significant difference in peptide response between the mutant W74F CGRP receptor and the WT receptor for either hAM or hαCGRP response (Figure 6.15). pEC$_{50}$ for hAM at the mutant receptor was 8.15± 0.07 compared to 8.04 ± 0.15 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=3). The E$_{max}$ values were 76.1 ± 4.32 for the mutant receptor and 76.8 ± 6.61 for the WT receptor, respectively (E$_{max}$ ± s.e.m.). hAM responses were: pEC$_{50}$ ± s.e.m., mutant 9.99 ± 0.15 vs WT 9.84 ± 0.12 and E$_{max}$ ± s.e.m., mutant 77.5 ± 6.43 vs WT 75.7 ± 5.58 (n=3).

**Figure 6.15** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant W74F CGRP receptor and the WT CGRP receptor. The graphs are representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.2.2.2.3 W74Y CGRP receptor

The hAM potency was significantly enhanced at the mutant receptor (Figure 6.16a). There was a ∼3-fold increase in pEC$_{50}$ value (pEC$_{50}$ ± s.e.m., mutant 8.55 ± 0.18 vs WT 8.05 ± 0.17, p < 0.01 by paired t-test, n=4) whereas the maximum response was not significantly altered (E$_{\text{max}}$ ± s.e.m., mutant 76.4 ± 2.60 vs WT 74.0 ± 2.99). On the other hand, there was no significant difference in hαCGRP response between the mutant W74Y CGRP and the WT receptor (Figure 6.16a) pEC$_{50}$ for hαCGRP at the mutant receptor was 9.98 ± 0.06 compared to 9.89 ± 0.09 for the WT receptor (pEC$_{50}$ ± s.e.m.; n=4). The E$_{\text{max}}$ values were 75.7 ± 4.71 for the mutant receptor and 74.2 ± 3.25 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.).

Figure 6.16 cAMP data for (a) hAM and (b) hαCGRP responses at the mutant W74Y CGRP receptor and the WT CGRP receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.2.2.4 W74A CGRP receptor

The hAM potency was significantly enhanced at the mutant receptor (Figure 6.17a). There was a ~3-fold increase in pEC$_{50}$ value (pEC$_{50}$ ± s.e.m., mutant 8.51 ± 0.21 vs WT 8.05 ± 0.17, $p < 0.01$ by paired t-test, n=4) whereas the maximum response was not significantly altered (E$_{\text{max}}$ ± s.e.m., mutant 75.5 ± 1.81 vs WT 74.0 ± 2.99). On the other hand, there was no significant difference in hαCGRP response between the mutant W74A CGRP and the WT receptor (Figure 6.17b) pEC$_{50}$ for hαCGRP at the mutant receptor was 10.1 ± 0.13 compared to 9.89 ± 0.09 at the WT receptor (pEC$_{50}$ ± s.e.m.; n=4). The E$_{\text{max}}$ values were 74.0 ± 3.62 for the mutant receptor and 74.2 ± 3.25 for the WT receptor, respectively (E$_{\text{max}}$ ± s.e.m.).

**Figure 6.17** cAMP data for (a) hAM and (b) hαCGRP responses at the mutant W74A CGRP receptor and the WT CGRP receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
6.2.2.2.2.5 W74N CGRP receptor

The hAM potency was significantly enhanced at the mutant receptor (Figure 6.18a). There was a ~4-fold increase in pEC\(_{50}\) value (pEC\(_{50}\) ± s.e.m., mutant 8.63 ± 0.21 vs WT 8.05 ± 0.17, \(p < 0.05\) by paired t-test, n=4) whereas the maximum response was not significantly altered (E\(_{\text{max}}\) ± s.e.m., mutant 75.7 ± 2.87 vs WT 74.0 ± 2.99). On the other hand, there was no significant difference in h\(\alpha\)CGRP response between the mutant W74N CGRP and the WT receptor (Figure 6.18b) pEC\(_{50}\) for h\(\alpha\)CGRP at the mutant receptor was 10.1 ± 0.14 compared to 9.89 ± 0.09 at the WT receptor (pEC\(_{50}\) ± s.e.m.; n=4). The E\(_{\text{max}}\) values were 76.6 ± 4.63 for the mutant receptor and 74.2 ± 3.25 for the WT receptor, respectively (E\(_{\text{max}}\) ± s.e.m.).

![Figure 6.18](image.png) cAMP data for (a) hAM and (b) h\(\alpha\)CGRP responses at the mutant W74N CGRP receptor and the WT CGRP receptor. The graphs are representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.
### 6.2.2.3 Summary of effects of amino acid substitutions at position 74 in RAMPs 1/3

**Table 6.1** Summary of pEC$_{50}$ values for human RAMP3 mutants at position 74, expressed with HA-CLR. Data are mean ± s.e.m. of 3-4 independent experiments, each in triplicate. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to WT by paired t-test.

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<th>Amino acid substitution</th>
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<th>hAM Mutant</th>
<th>haCGRP WT</th>
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Table 6.2 Summary of pEC$_{50}$ values for human RAMP1 mutants at position 74, expressed with HA-CLR. Data are mean ± s.e.m. of 3-4 independent experiments, each in triplicate. *$p < 0.05$ and **$p < 0.01$ compared to WT by paired t-test.

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<th>Native amino acid</th>
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<th>Amino acid substitution</th>
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<th>Mutant</th>
<th>hαCGRP WT</th>
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6.3 Discussion

The reciprocal effect produced by W74E RAMP1 and E74W RAMP3 in hAM potency in Chapters 4 and 5 suggested that E74 might be important for AM interactions with CLR. Therefore, this possibility was further explored through the use of a peptide fragment of AM, AM_{15-52}. AM_{15-52} exhibited similar behaviour to that of the full length AM. This provides insight into the residues of AM likely to be involved in interaction with E74, eliminating a role for the N-terminal 14 amino acids. In fact, a recent study using peptide chimaeras of CGRP and AM indicated that the last nine amino acids of AM at the C terminus might be responsible for interacting with or near E74 of RAMP3 (Robinson et al., 2009). This supports the first step that the C terminus of the peptide binds to the N terminus of the receptor to induce an affinity trap in the two domain binding mechanism that has been suggested for the family B GPCRs (Hoare, 2005).

AM2 (47 amino acids) response was also determined at the mutant receptors containing W74E RAMP1 and E74W RAMP3 to study whether the mode of action was conserved in this closest relative of AM. AM2 is produced from a different gene than AM and has only limited sequence identity (Refer to Chapter 1 Figure 1.1), but the mutations at 74 also induced a reciprocal effect in peptide potency. This suggests that E74 is crucial for different forms of AM interactions with the receptor and the potential region of AM2 that is involved in the interaction at/around position 74 may reside within the conserved regions/residues with AM. There are two regions that are more conserved between AM and AM2, the N-terminal residues 5-26 and the C-terminal residues 37-47 in AM2 (Refer to Chapter 1 Figure 1.1). It is more likely that the last a few residues at the C terminus are involved in the interactions at or around position 74 of RAMP3, as suggested for AM above. In addition, it is very interesting that AM2 appeared to be selective at the AM2 receptor over the CGRP receptor in this study; this is not consistent with the previously published studies where AM2 displayed equivalent potency across all RAMP/CLR complexes (Hay et al., 2005; Roh et al., 2004). The reason for this difference with the other studies is unclear. Different cells (HEK293T) used by Roh et al. might be a possible explanation, however, Cos 7 cells were also used by Hay et al. It seems that the nature of AM2 interactions with the receptors is certainly more complex than it appeared to be in the initial discovery and characterisation of this peptide. Furthermore, the reduction in AM2 potency at E74W AM2 receptors was of a smaller magnitude than the increased potency observed with this peptide at the W74E CGRP receptor, further emphasising that AM2 may have a different mode of interaction with the two receptors.
Like the phenomena observed in the cAMP assay, a reciprocal effect was also observed in the ¹²⁵I-hAM₁₃₋₅₂ radioligand binding assay at the E74W AM₂ and W74E CGRP receptors. Both affinity and maximum specific binding was significantly reduced at the E74W AM₂ receptor whereas these values were enhanced at the W74E CGRP receptor. It seems that E74 in RAMP3 is important for AM binding to the receptor and thus mutations introduced at this position affected both AM affinity and potency.

Further characterisation has also been performed at position 74 in RAMPs 1 and 3 through amino acid substitutions. There was a trend of decrease in cell-surface expression for the AM₂ receptor containing RAMP3 mutants but increase for the CGRP receptor containing RAMP1 mutants, however, none of these were significant. On the other hand, nearly all the amino acid substitutions at position 74 in either RAMP1 or RAMP3 had an impact on AM potency (Table 6.1 and 6.2). Residue swapping between the native residues at position 74 in RAMPs 1 and 3 showed a reciprocal effect on AM potency; the direction and magnitude of the shift in pEC₅₀ values were consistent with the data generated in Chapters 4 and 5. Similarly, other amino acid substitutions in RAMP3 and those in RAMP1 generally produced opposite effects; AM potency was mostly reduced at the AM₂ receptors containing the RAMP3 mutants, but enhanced at the CGRP receptors containing RAMP1 mutants. Some of these changes were accompanied by altered receptor cell-surface expression (although not significant) that might have contributed to the potency changes observed.

The most intriguing observation was the differential effects on AM potency induced by Phe and Tyr substitutions in RAMP3. Both Phe and Tyr contain an aromatic side chain, but only differ by a hydroxyl group present on the aromatic ring of Tyr. When the native E74 in RAMP3 was replaced with Trp which has a bulky aromatic side chain, significantly reduced AM potency was observed. Consistent with the Trp substitution, Phe substitution which also introduced a bulky aromatic residue to position 74 in RAMP3, also significantly reduced AM potency. However, when Tyr which is virtually a Phe residue with a hydroxyl group was introduced, the mutant AM₂ receptor generated an AM response not significantly different from the WT receptor. Therefore, although the presence of an aromatic ring seems to hinder the interaction at position 74 for high potency AM response, it can be partly rescued by a hydroxyl group which presumably restored an important bond required for AM interactions with the receptor. This argument is supported by the effects seen at the CGRP receptors containing Phe and Tyr substitutions in RAMP1. Replacing the native Trp in RAMP1 with Phe which also contains an aromatic ring did not significantly alter AM potency. However,
Tyr containing the hydroxyl group on the aromatic ring was able to significantly enhance AM potency.

The functional group in Glu contains a negatively charged carboxyl group, presumably forming a hydrogen bond and/or an electrostatic bond at position 74 which may be important for high potency AM response. The hydroxyl group in Tyr has a tendency to be ionised and therefore can potentially act as an acceptor for a hydrogen bond to be formed with the neighbouring residues from either AM or CLR at position 74. The consistent observations seen with Phe and Tyr substitutions in RAMPs 1 and 3 suggest that the hydroxyl group present in Tyr enables or mimics similar bond formation that is required for high potency AM response as at the native E74. Nevertheless, this interaction is not as strong as that formed by the native E74, likely due to the presence of the bulky aromatic ring in Tyr which may have disturbed the interaction involving the hydroxyl group. This may explain the slightly smaller effect on AM potency observed with Tyr substitution compared to Glu in both RAMPs 1 and 3.

To further investigate the potential importance of the bond formation by a hydroxyl group or its ionised form at position 74 in RAMP3, Ser and Thr substitutions that both contain a hydroxyl group were compared to Ala substitution which lacks any functional group in its side chain. Unlike the differential effects seen with Phe vs Tyr substitutions, Ala, Ser and Thr substitutions all reduced AM potency, despite the hydroxyl group present in Ser and Thr. It is likely that the functional groups that are potentially involved in the interaction at position 74 need to be in close proximity to allow bond formation, however, this could not be achieved in the case of Ser and Thr substitutions due to the small side chains that they contain. The data emphasise the importance of the size and geometry of the residue at position 74 for AM response.

As the size of the amino acid appeared to be important for the interaction at position 74 in RAMP3, substitutes of similar size and geometry to the native E74 were studied. The native E74 contains a negatively charged side chain, therefore it is not surprising to see a reduced AM potency when E74 was replaced by a positively charged amino acid, Arg. This is consistent with the reduced AM potency observed when Lys, also positively charged, was introduced to position 74 in RAMP3 to replace Glu in the AM2 receptor (Hay et al., 2006a). Furthermore, the strength of the bond formed at position 74 seems to be important. Asp substitution, a very similar amino acid to Glu, which also possesses a negatively charged
carboxyl group in its side chain, generated equivalent AM potency to the WT AM$_2$ receptor (Hay et al., 2006a). In contrast, reduced AM potency was seen with Gln substitution in the previous study (Hay et al., 2006a) and Asn substitution in this study despite that they are similar to Glu and Asp, respectively, in size and geometry. Although Gln and Asn have a polar amide group which can also act as an electron donor, they lack the negative charge that is present in Glu and Asp; thus the strength of the interaction formed may have been impaired and thus reduced AM potency.

On the other hand, amino acid substitutions introduced at position 74 in RAMPs 1 and 3 generally produced little effect on CGRP potency. Only two substitutions, Trp and Phe, introduced to RAMP3 showed small but statistically significant increases in CGRP potency. This general lack of effect is consistent with earlier studies where Asp, Gln and Lys substitutions in RAMP3 and Ala and Lys substitutions in RAMP1 at position 74 all showed WT-like CGRP response at the AM$_2$ or CGRP receptor, respectively (Hay et al., 2006a; Moore et al., 2010; Qi et al., 2008). Despite that W74 has been proposed to contribute to the CGRP binding site based on the crystal structure of the RAMP1 N terminus (Kusano et al., 2008), data generated in this study and previous studies (Hay et al., 2006a; Qi et al., 2008) together suggest that position 74 in RAMPs is not involved in CGRP to receptor interactions.

In conclusion, data generated in this study has further eliminated a role of position 74 in RAMP1 or RAMP3 in CGRP response, but emphasised on the importance of the native residue Glu at position 74 of RAMP3 for AM interaction. E74 seems to be sensitive to many amino acid substitutions. In addition, the interaction between AM and E74 of RAMP3 may be indirect, as only small magnitudes of changes were seen by amino acid substitutions. It is likely that E74 interacts with some residue in CLR so that the receptor is in the right conformation for AM to bind. Although the interaction environment around position 74 of the RAMP seems to be more complicated than just being majorly contributed by a specific side chain interaction, amino acid substitutions performed in this study have provided some insight to the interaction at position 74 in RAMP3. E74 of RAMP3 appears to be crucial for high potency AM response at the AM$_2$ receptor.
Chapter 7

Characterisation of a naturally occurring variant of the human calcitonin receptor

7.1 Introduction

The structure-function relationship has been determined for residues in RAMPs 1 and 3 in the previous chapters. Whilst it is clear that the RAMP N terminus plays an important role in determining receptor pharmacology, it is also important to study the receptor as it is the core GPCR that forms the major ligand binding domain and mediates the receptor signal transduction. However, compared to the RAMP residues, residues on the N-terminal domain of the receptor are less characterised (Chapter 1, Section 1.8.4). This is mainly due to the complexity in the receptor structure with a large number of residues involved and also the lack of structural information. In 1995, Albrandt and colleagues cloned a splice variant of the human calcitonin receptor from human breast carcinoma MCF-7 cells (Albrandt et al., 1995). The cloned receptor is a insert negative variant (hCT(a), lacking the 16 amino acid insert in the first intracellular domain) but with a additional truncation of the first 47 amino acids of its N terminus. This truncation includes the predicted signal sequence of 22 amino acids and one of the four potential N-linked glycosylation sites. Tissue distribution analysis identified the transcript of the truncated hCT(a) variant in various human tissues including kidney, skeletal muscle, lung, both the caudate nucleus and hypothalamus regions of the brain, whole brain and fetal brain (Albrandt et al., 1995). It was also faintly visible in the pancreas, but not in SK-N-MC neuroblastoma or U-2 OS osteogenic sarcoma cell lines.

Despite lacking the predicted signal peptid, this truncated hCT(a) variant seemed to still express at the cell surface and some limited pharmacological characterisation has been carried out with it. Cos 7 cells transfected with the truncated hCT(a) variant displayed high affinity binding for $^{125}$I sCT, but a lower $B_{\text{max}}$ compared to cells transfected with the full length hCT(a) (Albrandt et al., 1995). In addition, both the full length and truncated hCT(a) showed a potency order of sCT > hCT > hAmy (Albrandt et al., 1995). However, direct comparisons between the full length and truncated hCT(a) for these peptide potencies were not appropriate as these two forms of receptors were characterised in separate experiments in this study. Characterisation has also been performed on the truncated hCT(a) variant to determine the role of the three potential N-linked glycosylation sties. Alanine substitutions was
introduced to each or all three sites and the data suggested that the native Asn residues were important for sCT binding and potency (Ho et al., 1999). Nevertheless, these were some very early observations which were made before it was known that RAMPs are required for reconstituting the Amy receptor phenotypes. The Amy receptor phenotypes that could potentially result from the truncated hCT\textsubscript{(a)} and RAMP interactions have never been described or characterised. In addition, it has been demonstrated that amino acids 23-60 in the N terminus of mouse CLR mediates its interaction with mouse RAMP1 (Ittner et al., 2005); thus it is possible that the truncation may reduce its ability for RAMP association and the Amy receptor phenotype formation. Therefore, a study on the truncated form of hCT\textsubscript{(a)} has been carried out in this chapter with two objectives: 1. to determine the ability of the truncated hCT\textsubscript{(a)} variant to associate with RAMP1; 2. to pharmacologically characterise the truncated hCT\textsubscript{(a)} variant both with and without hRAMP1. These characterisations could potentially assign some specific roles to region 1-47 of hCT\textsubscript{(a)} and provide new insights to our understanding in ligand binding to the CT and Amy receptors.

7.2 Generation of the truncated form of hCT\textsubscript{(a)}

A truncation mutant construct of untagged hCT\textsubscript{(a)} (proline variant at 447) with 1-47 residues removed was obtained as a gift from Prof Laurence Miller, Mayo Clinic, Arizona, USA (Dong et al., 2009). Sequence analysis of the truncated hCT\textsubscript{(a)} variant identified in Albrandt et al. (Albrandt et al., 1995) shows that the isoform is a leucine variant at position 447 and also contains an isoleucine to threonine mutation at position 347. Therefore, two point mutations (I347T and P447L) were introduced into the untagged truncation mutant construct obtained from Prof Laurence Miller to generate a DNA construct that has the same amino acid sequence as the naturally occurring hCT\textsubscript{(a)} variant identified in the original publication (Albrandt et al., 1995). This construct will be denoted \(\Delta(1-47)hCT\textsubscript{(a)}\) in this chapter. An untagged version of hCT\textsubscript{(a)} which is also a Leu variant at position 447 was used as the WT control for all the characterisations performed with \(\Delta(1-47)hCT\textsubscript{(a)}\) in this chapter.

7.3 Cell-surface expression

Cell surface expression of hAMY\textsubscript{1(a)} receptor phenotype formed by \(\Delta(1-47)hCT\textsubscript{(a)}\) and mychRAMP1 was measured by detecting mychRAMP1 expression at the cell surface in whole-cell ELISA. Figure 7.1a shows the cell surface expression of the truncated form of hAMY\textsubscript{1(a)} Receptor \((\Delta(1-47)hCT\textsubscript{(a)}/\text{mychRAMP1})\) as a percentage of WT hAMY\textsubscript{1(a)} receptor \((hCT\textsubscript{(a)}/\text{mychRAMP1})\). The expression of \(\Delta(1-47)hAMY\textsubscript{1(a)}\) receptor was significantly
reduced \( (p < 0.001 \text{ by one-way ANOVA}) \), only achieving ~60% of WT AMY\(_1(a)\) receptor expression at the cell surface.

It is also important to analyse the cell surface expression of \( \Delta(1-47)hCT(a) \) to elucidate whether the reduced cell surface expression of the truncated hAMY\(_1(a)\) receptor was due to the impaired ability of \( \Delta(1-47)hCT(a) \) in associating with RAMP1 or simply caused by the reduced expression of hCT\(_(a)\) by the truncation. Therefore, an anti-CTR antibody (9B4) recognising the N-terminal domain of the hCT\(_(a)\) receptor beyond residue 47 was used to analyse the cell-surface expression of \( \Delta(1-47)hCT(a) \) and hCT\(_(a)\) both in complex with RAMP1 (Figure 7.1b) and alone (Figure 7.1c). As shown in Figure 7.1b, \( \Delta(1-47)hCT(a) \) expression was enhanced in complex with RAMP1 compared to hCT\(_(a)\) expression with RAMP1, however, this was not statistically significant. In addition, hCT\(_(a)\) expression was significantly reduced in the presence of RAMP1 compared to hCT\(_(a)\) alone \( (p < 0.05 \text{ by one-way ANOVA followed by Dunnett’s test}) \) (Figure 7.1b). On the other hand, \( \Delta(1-47)hCT(a) \) alone displayed a expression level which was comparable to hCT\(_(a)\) alone (Figure 7.1c).

![Figure 7.1](image.png)

**Figure 7.1** (a) Cell-surface expression of hAMY\(_1(a)\) (hCT\(_(a)\)/mycRAMP1) and \( \Delta(1-47)hAMY_1(a) \) (\( \Delta(1-47)hCT(a)/\text{mycRAMP1} \)), measuring mycRAMP1 expression. (b) Cell-surface expression of hCT\(_(a)\) and \( \Delta(1-47)hCT(a) \) in complex with mycRAMP1, measuring hCT\(_(a)\) expression. Expression of hCT\(_(a)\) alone at the cell surface was also included for comparison. (c) Cell-surface expression of hCT\(_(a)\) and \( \Delta(1-47)hCT(a) \) alone. Data are mean ± s.e.m. of three independent experiments, each performed with eight replicates; \(*p < 0.05\) and \(***p < 0.001\) by one-way ANOVA followed by Dunnett’s test.
The 9B4 anti-CTR antibody has an epitope at the N-terminal domain of hCT\textsubscript{(a)} beyond residue 47. This epitope position is possibly involved or close to the RAMP1 association site within hCT\textsubscript{(a)} as residues 23-60 at the N terminus of CLR have been demonstrated to be responsible for RAMP1 association (Ittner \textit{et al.}, 2005). Therefore it is possible that the apparently reduced hCT\textsubscript{(a)} expression observed in the presence of RAMP1 might have resulted from the masking of antibody epitope by RAMP1 association, which is removed with the 47 amino acid truncation. To investigate this possibility, HA-CT\textsubscript{(a)} expression was determined both in the absence and presence of RAMP1 using anti-HA antibody. The HA-tag locates at the extreme N terminus adjacent to the signal peptide in HA-CT\textsubscript{(a)}; it is thus less likely to be affected by RAMP1 association. As shown in Figure 7.2, HA-CT\textsubscript{(a)} expression was substantially higher in complex with RAMP1 compared to HA-CT\textsubscript{(a)} alone at the cell surface.

![Figure 7.2](image)

**Figure 7.2** Cell-surface expression of HA-CT\textsubscript{(a)} with and without mycRAMP1, measured using anti-HA antibody. Data shown are ratios of absorbance readings. The graph is representative of two independent experiments, each performed with eight replicates.
7.4 Functional characterisation using cAMP assays

Cos 7 cells were transfected with Δ(1-47)hCT(a) both in the presence and absence of mycRAMP1. The pharmacology of Δ(1-47)hCT(a) and Δ(1-47)hAMY1(a) were compared to their corresponding WT receptors, hCT(a) and hAMY1(a), respectively with agonists and antagonists. Several agonist and antagonist responses were determined in cAMP assays. The selected agonists include the cognate peptides for AMY and CTR receptors, rAmy and hCT, respectively and also sCT and three forms of CGRP: hαCGRP, hβCGRP and TyrºhαCGRP. All these three forms of CGRP have been demonstrated to display high potency at AMY1(a) receptors but not other CT(a) containing receptors (Hay et al., 2005). Thus, these peptides can be used as useful tools to determine the formation of AMY1(a) receptor phenotypes. In addition, antagonists including sCT8-32 and AC187 (agonist Amy) responses were measured.

7.4.1 Agonist pharmacology

7.4.1.1 rAmy response

The rAmy responses at the full length and truncated hAMY1(a) and hCT(a) receptors are presented and summarised in Figure 7.3 and Table 7.1. There was a small reduction in rAmy potency at Δ(1-47)hAMY1(a) compared to hAMY1(a), however, this difference was not statistically significant. On the other hand, the rAmy potency was significantly reduced at Δ(1-47)hCT(a) compared to hCT(a) (p < 0.05 by unpaired t-test), displaying a ~5-fold reduction. The rAmy responses were also compared between the AMY1(a) with CT(a) receptor phenotypes to determine the expected enhancement of rAmy potency at the AMY1(a) and Δ(1-47)hAMY1(a) receptors. rAmy was ~15-fold more potent at hAMY1(a) than hCT(a) (p < 0.01 by unpaired t-test) and ~22-fold more potent at Δ(1-47)hAMY1(a) than Δ(1-47)hCT(a) (p < 0.01 by unpaired t-test). In addition, the maximum response was not significantly different in any of the comparisons above.
**Figure 7.3** cAMP data for rAmy responses at hAMY$_{(a)}$, Δ(1-47)hAMY$_{(a)}$, hCT$_{(a)}$ and Δ(1-47)hCT$_{(a)}$. The graph is representative of four to five independent experiments. Data points are mean ± s.e.m. of triplicate assay points.

**Table 7.1** Summary of pEC$_{50}$ and $E_{\text{max}}$ for rAmy responses at hAMY$_{(a)}$, Δ(1-47)hAMY$_{(a)}$, hCT$_{(a)}$ and Δ(1-47)hCT$_{(a)}$ measured in cAMP assay. $^{@@} p < 0.01$ vs hCT$_{(a)}$; $^{$$} p < 0.01$ vs Δ(1-47)hCT$_{(a)}$; $^{*} p < 0.05$ vs hCT$_{(a)}$. Data were analysed using unpaired t-test.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>pEC$_{50}$ ± s.e.m.</th>
<th>$E_{\text{max}}$ ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAMY$_{(a)}$</td>
<td>10.5±0.18 $^{@@}$ (n=5)</td>
<td>73.3±4.22</td>
</tr>
<tr>
<td>Δ(1-47)hAMY$_{(a)}$</td>
<td>9.98±0.18 $^{$$}$ (n=5)</td>
<td>69.2±2.71</td>
</tr>
<tr>
<td>hCT$_{(a)}$</td>
<td>9.30±0.09 (n=4)</td>
<td>75.3±3.49</td>
</tr>
<tr>
<td>Δ(1-47)hCT$_{(a)}$</td>
<td>8.63±0.18 $^{*}$ (n=4)</td>
<td>74.0±2.88</td>
</tr>
</tbody>
</table>
7.4.1.2 hCT response

The hCT responses at the full length and truncated hAMY$_{1(a)}$ and hCT$_{(a)}$ receptors are presented and summarised in Figure 7.4 and Table 7.2. There was a significant ~25-fold reduction in hCT potency at Δ(1-47)hAMY$_{1(a)}$ compared to the full length hAMY$_{1(a)}$ ($p < 0.01$ by unpaired t-test). In addition, hCT potency was also significantly reduced at Δ(1-47)hCT$_{(a)}$ compared to hCT$_{(a)}$ ($p < 0.01$ by unpaired t-test), displaying a ~12-fold reduction. The hCT responses were also compared between the AMY$_{1(a)}$ with CT$_{(a)}$ receptor phenotypes. Whilst hCT was equally potent at both hAMY$_{1(a)}$ and hCT$_{(a)}$ receptors, there was also no significant difference in hCT potency for Δ(1-47)hAMY$_{1(a)}$ and Δ(1-47)hCT$_{(a)}$. The maximum response was not significantly different in any of the comparisons above.

![Figure 7.4](image-url)

**Figure 7.4** cAMP data for hCT responses at hAMY$_{1(a)}$, Δ(1-47)hAMY$_{1(a)}$, hCT$_{(a)}$ and Δ(1-47)hCT$_{(a)}$. The graph is representative of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.

**Table 7.2** Summary of pEC$_{50}$ and E$_{max}$ for hCT responses at hAMY$_{1(a)}$, Δ(1-47)hAMY$_{1(a)}$, hCT$_{(a)}$ and Δ(1-47)hCT$_{(a)}$ measured in cAMP assay. **$p < 0.01$ vs hAMY$_{1(a)}$; +$p < 0.01$ vs hCT$_{(a)}$. Data were analysed using unpaired t-test.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>hCT (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC$_{50}$ ± s.e.m.</td>
</tr>
<tr>
<td>hAMY$_{1(a)}$</td>
<td>10.6±0.35</td>
</tr>
<tr>
<td>Δ(1-47)hAMY$_{1(a)}$</td>
<td>9.19±0.07**</td>
</tr>
<tr>
<td>hCT$_{(a)}$</td>
<td>10.7±0.09</td>
</tr>
<tr>
<td>Δ(1-47)hCT$_{(a)}$</td>
<td>9.66±0.21**</td>
</tr>
</tbody>
</table>
7.4.1.3 sCT response

The sCT responses at the full length and truncated hAMY_{1(a)} and hCT_{(a)} receptors are presented and summarised in Figure 7.5 and Table 7.3. hAMY_{1(a)}, Δ(1-47)hAMY_{1(a)}, hCT_{(a)} and Δ(1-47)hCT_{(a)} all showed equivalent potencies to sCT. There was no significant difference for any comparison group except the maximum response was slightly reduced at Δ(1-47)hAMY_{1(a)} compared to Δ(1-47)hCT_{(a)} (p < 0.05 by unpaired t-test).

![Figure 7.5](image)

**Figure 7.5** cAMP data for sCT responses at hAMY_{1(a)}, Δ(1-47)hAMY_{1(a)}, hCT_{(a)} and Δ(1-47)hCT_{(a)}. The graph is representative of five independent experiments. Data points are mean ± s.e.m. of triplicate assay points.

**Table 7.3** Summary of pEC$_{50}$ and E$_{max}$ for sCT responses at hAMY$_{1(a)}$, Δ(1-47)hAMY$_{1(a)}$, hCT$_{(a)}$ and Δ(1-47)hCT$_{(a)}$ measured in cAMP assay. $^\dagger$ p < 0.05 vs Δ(1-47)hCT$_{(a)}$. Data were analysed using unpaired t-test.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>sCT (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC$_{50}$ ± s.e.m.</td>
</tr>
<tr>
<td>hAMY$_{1(a)}$</td>
<td>11.1±0.08</td>
</tr>
<tr>
<td>Δ(1-47)hAMY$_{1(a)}$</td>
<td>10.8±0.20</td>
</tr>
<tr>
<td>hCT$_{(a)}$</td>
<td>10.9±0.24</td>
</tr>
<tr>
<td>Δ(1-47)hCT$_{(a)}$</td>
<td>10.9±0.09</td>
</tr>
</tbody>
</table>
7.4.1.4 hαCGRP response

The hαCGRP responses at the full length and truncated hAMY_{1(a)} and hCT_{(a)} receptors are presented and summarised in Figure 7.6 and Table 7.4. There was a ~4-fold increase in hαCGRP potency at Δ(1-47)hAMY_{1(a)} compared to hAMY_{1(a)} (p < 0.05 by unpaired t-test). On the other hand, hαCGRP potency was significantly reduced at Δ(1-47)hCT_{(a)} compared to hCT_{(a)} (p < 0.01 by unpaired t-test), displaying a ~7-fold reduction. The hαCGRP responses were also compared between the AMY_{1(a)} with CT_{(a)} receptor phenotypes to determine the expected enhancement in potency at the AMY_{1(a)} and Δ(1-47)hAMY_{1(a)} receptors. hαCGRP was ~66-fold more potent at hAMY_{1(a)} than hCT_{(a)} (p < 0.001 by unpaired t-test) and ~1862-fold more potent at Δ(1-47)hAMY_{1(a)} than Δ(1-47)hCT_{(a)} (p < 0.001 by unpaired t-test). The maximum response was not significantly different in any of the comparisons above.

![Graph](image)

**Figure 7.6** cAMP data for hαCGRP responses at hAMY_{1(a)}, Δ(1-47)hAMY_{1(a)}, hCT_{(a)} and Δ(1-47)hCT_{(a)}. The graph is representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.

**Table 7.4** Summary of pEC_{50} and E_{max} for hαCGRP responses at hAMY_{1(a)}, Δ(1-47)hAMY_{1(a)}, hCT_{(a)} and Δ(1-47)hCT_{(a)} measured in cAMP assay. *p < 0.05 vs hAMY_{1(a)}; ††††p < 0.001 vs hCT_{(a)}; †‰‰p < 0.01 vs Δ(1-47)hCT_{(a)}; †††p < 0.05 vs hCT_{(a)}. Data were analysed using unpaired t-test.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>hαCGRP (n=3)</th>
<th>pEC_{50} ± s.e.m.</th>
<th>E_{max} ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAMY_{1(a)}</td>
<td></td>
<td>10.2±0.17††††</td>
<td>69.8±3.83</td>
</tr>
<tr>
<td>Δ(1-47)hAMY_{1(a)}</td>
<td></td>
<td>10.8±0.09*‰‰‰</td>
<td>72.0±1.69</td>
</tr>
<tr>
<td>hCT_{(a)}</td>
<td></td>
<td>8.34±0.09</td>
<td>80.3±2.84</td>
</tr>
<tr>
<td>Δ(1-47)hCT_{(a)}</td>
<td></td>
<td>7.48±0.10**</td>
<td>76.5±0.34</td>
</tr>
</tbody>
</table>
7.4.1.5 hβCGRP response

The hβCGRP responses at the full length and truncated hAMY1(a) and hCT(a) receptors are presented and summarised in Figure 7.7 and Table 7.5. hβCGRP was equally potent at both Δ(1-47)hAMY1(a) and hAMY1(a). On the other hand, hβCGRP potency was significantly reduced at Δ(1-47)hCT(a) compared to hCT(a) \( (p < 0.05 \text{ by unpaired t-test}) \), displaying a ~5-fold reduction. The hβCGRP responses were also compared between the AMY1(a) with CT(a) receptor phenotypes to determine the expected enhancement in potency at the AMY1(a) and Δ(1-47)hAMY1(a) receptors. hβCGRP was ~37-fold more potent at hAMY1(a) than hCT(a) \( (p < 0.001 \text{ by unpaired t-test}) \) and ~126-fold more potent at Δ(1-47)hAMY1(a) than Δ(1-47)hCT(a) \( (p < 0.001 \text{ by unpaired t-test}) \). In addition, the maximum response was not significantly different in any of the comparisons above.

Figure 7.7 cAMP data for hβCGRP responses at hAMY1(a), Δ(1-47)hAMY1(a), hCT(a) and Δ(1-47)hCT(a). The graph is representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.

Table 7.5 Summary of pEC\(_{50}\) and \( E_{\text{max}} \) for hβCGRP responses at hAMY1(a), Δ(1-47)hAMY1(a), hCT(a) and Δ(1-47)hCT(a) measured in cAMP assay. \(@@@ p < 0.001 \text{ vs hCT(a)}; ^{\text{SS}} p < 0.01 \text{ vs Δ(1-47)hCT(a)}; ^{+} p < 0.05 \text{ vs hCT(a).} \) Data were analysed using unpaired t-test.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>hβCGRP (n=3)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC(_{50}) ± s.e.m.</td>
<td>( E_{\text{max}} ) ± s.e.m.</td>
</tr>
<tr>
<td>hAMY1(a)</td>
<td>10.6±0.12@@@</td>
<td>78.3±2.62</td>
</tr>
<tr>
<td>Δ(1-47)hAMY1(a)</td>
<td>10.4±0.24SS</td>
<td>74.7±2.98</td>
</tr>
<tr>
<td>hCT(a)</td>
<td>9.03±0.11</td>
<td>80.8±3.02</td>
</tr>
<tr>
<td>Δ(1-47)hCT(a)</td>
<td>8.34±0.18+</td>
<td>74.6±2.46</td>
</tr>
</tbody>
</table>
7.4.1.6 Tyr°hαCGRP response

The Tyr°hαCGRP responses at the full length and truncated hAMY_{1(a)} and hCT_{(a)} receptors are presented and summarised in Figure 7.8 and Table 7.6. Tyr°hαCGRP was equally potent at both Δ(1-47)hAMY_{1(a)} and hAMY_{1(a)}. On the other hand, Tyr°hαCGRP potency was significantly reduced at Δ(1-47)hCT_{(a)} compared to hCT_{(a)} \( (p < 0.05 \text{ by unpaired t-test}) \), displaying a ~3-fold reduction. The Tyr°hαCGRP responses were also compared between the AMY_{1(a)} with CT_{(a)} receptor phenotypes to determine the expected enhancement in potency at the AMY_{1(a)} and Δ(1-47)hAMY_{1(a)} receptors. Tyr°hαCGRP was ~214-fold more potent at hAMY_{1(a)} than hCT_{(a)} \( (p < 0.001 \text{ by unpaired t-test}) \) and ~1047-fold more potent at Δ(1-47)hAMY_{1(a)} than Δ(1-47)hCT_{(a)} \( (p < 0.001 \text{ by unpaired t-test}) \). The maximum response was not significantly different in any of the comparisons above.

Figure 7.8 cAMP data for Tyr°hαCGRP responses at hAMY_{1(a)}, Δ(1-47)hAMY_{1(a)}, hCT_{(a)} and Δ(1-47)hCT_{(a)}. The graph is representative of three independent experiments. Data points are mean ± s.e.m. of triplicate assay points.

Table 7.6 Summary of pEC_{50} and E_{max} for Tyr°hαCGRP responses at hAMY_{1(a)}, Δ(1-47)hAMY_{1(a)}, hCT_{(a)} and Δ(1-47)hCT_{(a)} measured in cAMP assay. @@ @@ @@ \( p < 0.001 \text{ vs } hCT_{(a)} \); $$$ $$$ $$$ \( p < 0.001 \text{ vs } Δ(1-47)hCT_{(a)} \); + \( p < 0.05 \text{ vs } hCT_{(a)} \). Data were analysed using unpaired t-test.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Tyr°hαCGRP (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC_{50} ± s.e.m.</td>
</tr>
<tr>
<td>hAMY_{1(a)}</td>
<td>9.96±0.10</td>
</tr>
<tr>
<td>Δ(1-47)hAMY_{1(a)}</td>
<td>10.1±0.15</td>
</tr>
<tr>
<td>hCT_{(a)}</td>
<td>7.63±0.13</td>
</tr>
<tr>
<td>Δ(1-47)hCT_{(a)}</td>
<td>7.09±0.12</td>
</tr>
</tbody>
</table>
7.4.2 Antagonist pharmacology

The $\Delta(1-47)\text{hCT}(a)$ and $\Delta(1-47)\text{hAMY}_1(a)$ receptors were also characterised with antagonists $\text{sCT}_{8-32}$ and AC187 using rAmy as the agonist. $\text{sCT}_{8-32}$ is a peptide fragment of sCT and AC187 is a fusion protein made by replacing the last three residues of sCT$_{8-32}$ with residues 35-37 from rAmy. Both peptides act as antagonists at AMY$_1(a)$ and CT$_1(a)$ receptors (Hay et al., 2005). Intracellular cAMP accumulation was measured for rAmy in the absence and presence of three antagonist concentrations (1 μM, 100 nM and 10 nM). Global Schild analysis was used to fit curves to the data points. F-test was conducted to compare if the Schild slope of the curves was significantly different to 1. In most analyses, the Schild slope was not different to 1. Therefore the Schild slope was constrained to a value of 1. The resulting estimate of $pA_2$ represents the $pK_B$.

7.4.2.1 sCT$_{8-32}$ antagonism

The rAmy responses antagonised by sCT$_{8-32}$ at the full length and truncated hAMY$_1(a)$ and hCT$_1(a)$ receptors are presented in Figure 7.9 and the $pK_B$ values are summarised in Table 7.7. sCT$_{8-32}$ produced right-ward shifts in the concentration-response curve for rAmy with no suppression of maximum response at all receptors. There was a small reduction in $pK_B$ at $\Delta(1-47)\text{hAMY}_1(a)$ compared to hAMY$_1(a)$, however it was not statistical significant. sCT$_{8-32}$ antagonism was also significantly reduced at $\Delta(1-47)\text{hCT}_1(a)$ compared to hCT$_1(a)$ ($p < 0.01$ by unpaired t-test), displaying a ~8-fold reduction in $pK_B$. In addition, sCT$_{8-32}$ antagonism was compared between the AMY$_1(a)$ and CT$_1(a)$ receptor phenotypes. sCT$_{8-32}$ was ~7-fold less potent an antagonist of hAMY$_1(a)$ compared to hCT$_1(a)$ ($p < 0.05$ by unpaired t-test). $pK_B$ for sCT$_{8-32}$ was also slightly lower at $\Delta(1-47)\text{hAMY}_1(a)$ compared to $\Delta(1-47)\text{hCT}_1(a)$, however, the reduction was not statistically significant.
Figure 7.9 cAMP data for rAmy responses in the presence of three concentrations of sCT8-32 at (a) hAMY1(a), (b) Δ(1-47)hAMY1(a), (c) hCT(a) and (d) Δ(1-47)hCT(a). The graphs are representatives of three to four to independent experiments. Data points are mean ± s.e.m. of triplicate assay points.

Table 7.7 pK_B values for sCT8-32 in antagonising rAmy responses at hAMY1(a), Δ(1-47)hAMY1(a), hCT(a) and Δ(1-47)hCT(a) measured in cAMP assay. *p < 0.05 vs hCT(a); **p < 0.01 vs hCT(a). Data were analysed using unpaired t-test.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>pK_B ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAMY1(a)</td>
<td>8.08 ± 0.28** (n=3)</td>
</tr>
<tr>
<td>Δ(1-47)hAMY1(a)</td>
<td>7.57± 0.31 (n=3)</td>
</tr>
<tr>
<td>hCT(a)</td>
<td>8.95 ± 0.17 (n=4)</td>
</tr>
<tr>
<td>Δ(1-47)hCT(a)</td>
<td>8.03 ± 0.12** (n=4)</td>
</tr>
</tbody>
</table>
Chapter 7

7.4.2.2 AC187 antagonism

The rAmy responses antagonised by AC187 at the full length and truncated hAMY$_{1(a)}$ and hCT$_{(a)}$ receptors are presented in Figure 7.10 and the pK$_B$ values are summarised in Table 7.8. AC187 produced right-ward shifts in the concentration-response curve for rAmy with no suppression of maximum response at all receptors. There was a significant ~7-fold reduction in pK$_B$ for AC187 at Δ(1-47)hAMY$_{1(a)}$ compared to hAMY$_{1(a)}$ ($p < 0.01$ by unpaired t-test). AC187 antagonism was also significantly reduced at Δ(1-47)hCT$_{(a)}$ compared to hCT$_{(a)}$ ($p < 0.01$ by unpaired t-test), displaying a ~11-fold reduction in pK$_B$. In addition, AC187 antagonism was compared between the AMY$_{1(a)}$ and CT$_{(a)}$ receptor phenotypes. pK$_B$ for AC187 was equivalent at Δ(1-47)hAMY$_{1(a)}$ compared to Δ(1-47)hCT$_{(a)}$, however, the reduction was not statistically significant. AC187 was ~3-fold more potent an antagonist of Δ(1-47)hAMY$_{1(a)}$ receptor compared to Δ(1-47)hCT$_{(a)}$ ($p < 0.05$ by unpaired t-test).
Figure 7.10 cAMP data for rAmy responses antagonised by three concentrations of AC187 at (a) hAMY\(_{1(a)}\), (b) \(\Delta(1-47)\)hAMY\(_{1(a)}\), (c) hCT\(_{(a)}\) and (d) \(\Delta(1-47)\)hCT\(_{(a)}\). The graphs are representatives of four independent experiments. Data points are mean ± s.e.m. of triplicate assay points.

Table 7.8 pK\(_B\) values for AC187 in antagonising rAmy responses at hAMY\(_{1(a)}\), \(\Delta(1-47)\)hAMY\(_{1(a)}\), hCT\(_{(a)}\) and \(\Delta(1-47)\)hCT\(_{(a)}\) measured in cAMP assay. **p < 0.01 vs hAMY\(_{1(a)}\); *p < 0.05 vs \(\Delta(1-47)\)hCT\(_{(a)}\); ++p < 0.01 vs hCT\(_{(a)}\). Data were analysed using unpaired t-test.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>pK(_B) (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAMY(_{1(a)})</td>
<td>9.25±0.07</td>
</tr>
<tr>
<td>(\Delta(1-47))hAMY(_{1(a)})</td>
<td>8.43±0.14*</td>
</tr>
<tr>
<td>hCT(_{(a)})</td>
<td>8.85±0.16</td>
</tr>
<tr>
<td>(\Delta(1-47))hCT(_{(a)})</td>
<td>7.82±0.16*</td>
</tr>
</tbody>
</table>
7.5 Role of I347T mutation

An Ile to Thr point mutation was identified at position 347 in the original sequence of the truncated hCT\textsubscript{(a)} variant cloned by Albrandt \textit{et al.} (Albrandt \textit{et al.}, 1995), but the contribution of this substitution to variant phenotype has not been analysed. Therefore it is important to demonstrate the effect of the I347T mutation on receptor expression and function in order to determine what contribution this mutation may make to Δ(1-47)hCT\textsubscript{(a)} and Δ(1-47)AMY\textsubscript{1(a)} phenotype. To test this, Δ(1-47)hCT\textsubscript{(a)}-I347 was made; this construct has the identical amino acid sequence as Δ(1-47)hCT\textsubscript{(a)} that has been used for characterisation in this chapter, except that it has an Ile residue at position 347 instead of Thr. The cell-surface expression of Δ(1-47)hCT\textsubscript{(a)}-I347 both in the presence and absence of RAMP1 was determined and rAmy and sCT responses were compared at the AMY\textsubscript{1(a)} receptor phenotypes formed by Δ(1-47)hCT\textsubscript{(a)}-I347 and Δ(1-47)hCT\textsubscript{(a)}.

7.5.1 Cell-surface expression

To determine the effect of I347 on receptor expression, RAMP1 translocation was measured and cell-surface expression of the different forms of CT\textsubscript{(a)} in the presence and absence of RAMP1 was also compared. mycRAMP1 expression was measured to give an indication of the AMY\textsubscript{1(a)} receptor expression at the cell surface. Δ(1-47)-I347hAMY\textsubscript{1(a)} expression was significantly reduced compared to AMY\textsubscript{1(a)}, but was slightly higher than Δ(1-47)hAMY\textsubscript{1(a)} (one-way ANOVA followed by Tukey’s test) (Figure 7.11a). In addition, Δ(1-47)hCT\textsubscript{(a)}-I347 expression was also determined both in the presence (Figure 7.11b) and absence of RAMP1 (Figure 7.11c). Δ(1-47)hCT\textsubscript{(a)}-I347 expression was significantly enhanced compared to the full length hCT\textsubscript{(a)} expression both with and without RAMP1 (one-way ANOVA followed by Tukey’s test). Δ(1-47)hCT\textsubscript{(a)}-I347 also showed higher expression level than Δ(1-47)hCT\textsubscript{(a)} in both cases, however, statistically significance was not achieved (one-way ANOVA followed by Tukey’s test).
Figure 7.11 (a) Cell-surface expression of three forms of hCT\(_{(a)}\)/mycRAMP1 complexes measuring mycRAMP1 expression. (b) Cell-surface expression of three forms of hCT\(_{(a)}\) with mycRAMP1, measuring hCT\(_{(a)}\) expression. (c) Cell-surface expression of three forms of hCT\(_{(a)}\) on their own. Data are mean ± s.e.m. of three independent experiments, each performed with eight replicates. **p < 0.01 and ***p < 0.001 vs hCT\(_{(a)}\)/mycRAMP1 (a &b) and *p < 0.05 vs hCT\(_{(a)}\) alone (c) by one-way ANOVA followed by Tukey’s test.
7.5.2 cAMP assay

rAmy and sCT responses were compared to determine the effect of the I347T mutation on receptor function. There was no apparent difference in either rAmy (Figure 7.12a) or sCT (Figure 7.12b) response between the hAMY\textsubscript{1(a)} receptors formed by mycRAMP1 and Δ(1-47)hCT\textsubscript{(a)}-I347 or Δ(1-47)hCT\textsubscript{(a)}.

![Graph (a)](image)

**Figure 7.12** cAMP data for (a) rAmy and (b) sCT responses at the hAMY\textsubscript{1(a)} receptors formed by mycRAMP1 and Δ(1-47)hCT\textsubscript{(a)}-I347 or Δ(1-47)hCT\textsubscript{(a)}. The graphs are from a single experiment, each data point representing the mean ± s.e.m. of triplicate assay points.
7.6 Discussion

Despite lacking the first 47 residues in the N terminus, the cell surface expression data suggest that Δ(1-47)hCT(a) is still capable of associating with RAMP1 to constitute an AMY1(a) receptor phenotype. However, the expression of Δ(1-47)hAMY1(a) was significantly reduced by ~40% compared to the full length hAMY1(a) (Figure 7.1a). In order to determine whether the reduced expression of Δ(1-47)hAMY1(a) at the cell surface was caused by the disrupted receptor to RAMP1 association or resulted from any reduction in hCT(a) expression, hCT(a) expression was in addition measured. Δ(1-47)hCT(a) showed higher cell-surface expression in complex with RAMP1 than hCT(a) with RAMP1. Unexpectedly, hCT(a) showed a significantly reduced expression when complexed with RAMP1 than alone at the cell surface; this may be caused by the possible masking of the antibody epitope on hCT(a) by the presence of RAMP1 as the cell surface expression data showed that HA-CT(a) expression was not reduced by RAMP1. Nevertheless, Δ(1-47)hCT(a) either with or without RAMP1 had a expression level which was comparable to that of hCT(a) alone (Figure 7.1b & c), indicating that the reduction seen in Δ(1-47)hAMY1(a) expression measured by RAMP1 was resulted from the possible disrupted receptor to RAMP1 association by the removal of residues 1-47. Some residues of hCT(a) involved in RAMP1 interaction might have been lost with the 47 amino acid truncation but not all of them, such that RAMP1 association is reduced but not completely lost. This observation is consistent with a previous study using CLR and PTH receptor chimaeras showing that N-terminal residues 23-60 of CLR were important for CLR and RAMP1 association (Ittner et al., 2005). Thus, it is likely that the region responsible for RAMP1 association lies closely beyond residue 47 of hCT(a), however, further study is required to determine the exact region involved.

It is interesting that Δ(1-47)hCT(a), although it lacks the signal peptide, was fully expressed at the cell surface. Signal peptides are short petide chains upstream of the proteins that direct the transport of the proteins. They direct the proteins to the cell surface in the case of receptors. They may get cleaved from the proteins after reaching to the cell surface. The underlying mechanism of how Δ(1-47)hCT(a) without the signal peptide was translocated to the cell surface is unclear. However, the signal peptide region of hCT(a) is predicted, it is possible that some regions/residues in other parts of the protein may also be involved as the recognition sequence for its translocation.
The formation of the hAMY1(a) receptor phenotype by Δ(1-47)hCT(a) and RAMP1 was further evidenced in the functional assays. A total of six peptide agonist responses were determined at Δ(1-47)hCT(a) both with and without RAMP1 (Figure 7.13). The differential peptide responses seen at hAMY1(a) vs hCT(a) were consistent with previously published data (Hay et al., 2005): rAmy, CGRP and hCT were approximately equally potent at hAMY1(a) whilst hCT was more potent than rAmy or CGRP at hCT(a). Peptide potencies for rAmy, hαCGRP, hβCGRP and Tyr°hαCGRP were all enhanced at Δ(1-47)hAMY1(a) compared to Δ(1-47)hCT(a), indicating that Δ(1-47)hCT(a) still associates with RAMP1 and forms a functional hAMY1(a) receptor.

**Figure 7.13** Summary of pEC50 values for the six peptide responses determined at hAMY1(a), Δ(1-47)hAMY1(a), hCT(a) and Δ(1-47)hCT(a). The statistics shown in red on the x-axis label are generated from comparisons of hAMY1(a) vs hCT(a) and Δ(1-47)hAMY1(a) vs Δ(1-47)hCT(a). NS, non-significant, *p < 0.05, **p < 0.01 and ***p < 0.001 by unpaired t-test.

hCT showed a significantly reduced potency at Δ(1-47)hCT(a) compared to hCT(a), suggesting that a region or residue important for interactions might have been lost. This is consistent with a previous investigation where residue T30 in the N terminus of hCT(a) was suggested to be interacting with position 26 in hCT (Dong et al., 2004a). The removal of T30 in Δ(1-47)hCT(a) would have disrupted a potential ligand (hCT) interaction site in the receptor and
resulted in reduced affinity and thus peptide potency. Like hCT, there was a general reduction in peptide potency for rAmy, hαCGRP, hβCGRP and TyrºhαCGRP at Δ(1-47)hCT(a) compared to hCT(a). It is clear that these reductions were not caused by reduced receptor expression at the cell surface as Δ(1-47)hCT(a) showed equivalent expression level to hCT(a) (Figure 7.1c). Thus, there may be one or more interaction sites within the first 47 amino acids of hCT(a) that are important for these peptide potencies, or the truncation has introduced some changes to the receptor conformation which affected the peptide to receptor interactions.

Nevertheless, further characterisation of Δ(1-47)hCT(a) in the presence of RAMP1 showed very interesting observations. rAmy, hαCGRP, hβCGRP and TyrºhαCGRP potencies, which were reduced at Δ(1-47)hCT(a), were restored at Δ(1-47)hAMY1(a); all the agonists showed equivalent or even higher peptide potencies at Δ(1-47)hAMY1(a) compared to hAMY1(a) (Figure 7.13). It seems that the presence of RAMP1 rescued rAmy and CGRP potencies. This phenomenon has never been reported before. It is possible that RAMP1 may have a role in stabilising these peptide to receptor interactions either by directly providing some binding sites for these ligands or changing hCT(a) conformation upon association to allow high affinity binding of rAmy and hCGRP.

In contrast, the restored potency for rAmy and CGRP was not observed for hCT at the full length and truncated AMY1(a); a significant reduction in potency was instead seen. The presence of RAMP1 did not seem to affect hCT potency in the assays performed in this chapter as hCT was equally potent at the full length hCT(a) and AMY1(a). This may suggest that the binding pocket of hCT might be different to those of rAmy and CGRPs on hCT(a). RAMP1 association with hCT(a) may have little impact on the hCT interactions with the receptor. This is consistent with the observation that RAMP is not needed for hCT to bind to hCT(a); a previous study has reported a slight reduction in hCT affinity (by ~2-fold) at the RAMP1/hCT(a) complex compared to hCT(a) alone (Christopoulos et al., 1999).

In the characterisation performed in this chapter, sCT displayed high and equivalent potency at the truncated and full length hCT(a) and AMY1(a) receptors. As sCT is known as a very potent agonist (Hilton et al., 2000; Poyner et al., 2002), it is possible that the truncation of residues 1-47 in hCT(a) did not produce an effect big enough to alter the very high potency of sCT. Therefore the affinity of sCT8-32 and AC187, which are not as high in affinity as sCT, were determined at Δ(1-47)hCT(a) with and without RAMP1. Of note, the potency of rAmy alone in the experiment with sCT8-32 (Figure 7.9) and AC187 (Figure 7.10) differed by more
than 10-fold. This amount of variation is commonly seen in the cell line studies using transient transfection. Nevertheless, it does not affect the interpretation of the data as an appropriate control was included in all the assays performed in this thesis. Broadly consistent with the published data (Hay et al., 2005), sCT8-32 was a more potent antagonist at hCT\textsubscript{(a)} compared to hAMY\textsubscript{1(a)} and AC187 showed higher affinity at hAMY\textsubscript{1(a)} compared to hCT\textsubscript{(a)} (Figure 7.14). On the other hand, both peptides were lower in affinity at the truncated receptors compared to the full length hCT\textsubscript{(a)} and hAMY\textsubscript{1(a)} receptors (of note, the statistical significance was not achieved for sCT8-32 at hAMY\textsubscript{1(a)}, one more experiment (n=4), as with the others, may have resulted a significant difference), suggesting that sCT potency might have also been reduced by the removal of the first 47 residues in the N terminus of hCT\textsubscript{(a)}. Although it has been suggested that there are differences in the binding sites for sCT and hCT on hCT\textsubscript{(a)} (Dong et al., 2004b; Pham et al., 2005), the peptide to receptor interactions are consistent in the sense of not being affected by RAMP1. sCT8-32 and AC187 displayed differential affinity at Δ(1-47)hCT\textsubscript{(a)} and Δ(1-47)hAMY\textsubscript{1(a)} (Figure 7.14), which were consistent with the full length receptors, providing additional evidence for the formation of hAMY\textsubscript{1(a)} receptor phenotype by the Δ(1-47)hCT\textsubscript{(a)} with RAMP1.

In addition, the data generated with sCT\textsubscript{8-32} and AC187 suggested that the last three residues of rAmy may not be involved in the interactions with either RAMP1 or the N-terminal domain 1-47. rAmy potency was restored by the cotransfection of RAMP1; AC187 which is essentially sCT\textsubscript{8-29} plus rAmy\textsubscript{35-37}, its affinity was reduced in the presence of RAMP1. It is thus likely that residues rAmy\textsubscript{35-37} may not be involved in the interaction with RAMP1 that could potentially contribute to the ligand binding. Furthermore, AC187 displayed higher affinity at hAMY\textsubscript{1(a)} compared to sCT\textsubscript{8-32}. This enhancement is presumably introduced by residues rAmy\textsubscript{35-37} in AC187. This order of affinity was conserved at Δ(1-47)hAMY\textsubscript{1(a)} for sCT\textsubscript{8-32} and AC187, suggesting that the truncation of 1-47 did not affect the interactions of the residues Amy\textsubscript{35-37} with the receptors.
Figure 7.14 pK_B for sCT_{8-32} and AC187 in antagonising rAmy at hAMY_{1(a)}, Δ(1-47)hAMY_{1(a)}, hCT_{(a)} and Δ(1-47)hCT_{(a)}. The statistics shown in red on the x-axis label are generated from comparisons hAMY_{1(a)} vs hCT_{(a)} and Δ(1-47)hAMY_{1(a)} vs Δ(1-47)hCT_{(a)}. NS, non-significant, *p < 0.05, and **p < 0.01 by unpaired t-test.

Furthermore, the effect of I347T mutation identified in the sequence of the truncated hCT_{(a)} variant published by Albrandt et al. (Albrandt et al., 1995) was also determined in this chapter. Similar to Δ(1-47)hAMY_{1(a)}, the cell-surface expression measured by mycRAMP1 of Δ(1-47)-I347hAMY_{1(a)} was also significantly reduced compared to the WT AMY_{1(a)} receptor, displaying a slightly higher expression level than Δ(1-47)hAMY_{1(a)}. In addition, the cell-surface expression of Δ(1-47)hCT_{(a)} was apparently enhanced both with and without RAMP1 when this point mutation was introduced, however, the increase in expression was not statistically significant in either case. On the other hand, the I347T mutation had little effect in Δ(1-47)hAMY_{1(a)} receptor function; both rAmy and sCT responses were not affected by this mutation. Therefore, the I347T mutation found in Δ(1-47)hCT_{(a)} may have increased receptor expression slightly at the cell surface, but does not seem to affect Δ(1-47)hAMY_{1(a)} receptor function. Nevertheless, the proline residues close by in the 6th TM domain of hCT_{(a)} have been suggested to be important in peptide binding and potency. Alanine substitutions at P326 and P336 in hCT_{(a)} led to a significant reduction in hCT potency and little or no ^{125}\text{I}\text{-}hCT binding without affecting the cell-surface expression of the receptor (Bailey & Hay, 2007). However, the effect seemed to be specific to hCT as sCT potency and affinity were not affected. This lack of effect in sCT potency is actually consistent with the data generated in this thesis. The corresponding residues in CLR have also been mutated to alanine (Conner...
et al., 2005). P321A and P331A substitutions in CLR caused a reduced CGRP potency and affinity in complex with RAMP1, but the cell-surface expression of the mutant CGRP receptors was not affected.

In conclusion, despite lacking the first 47 amino acids in the N terminus, Δ(1-47)hCT\textsubscript{(a)} retains its ability to associate with RAMP1 and thus form a functional hAMY\textsubscript{1(a)} receptor phenotype. Though the truncation appears to reduce hAMY\textsubscript{1(a)} expression at the cell surface, it does not seem to have any significant effect on hAMY\textsubscript{1(a)} function. On the other hand, peptide potencies (except sCT) were all reduced at Δ(1-47)hCT\textsubscript{(a)}, suggesting that residues 1-47 may contain one or more interaction sites that are important for peptide responses at hCT\textsubscript{(a)}. Very interestingly, the reduced potency seen at Δ(1-47)hCT\textsubscript{(a)} could be restored by the presence of RAMP1 for rAmy and CGRP but not hCT. This may indicate that RAMP1 has differential roles in these peptide interactions with hAMY\textsubscript{1(a)}. 


Chapter 8
Discussion and Conclusion

8.1 Overview

This thesis has a focus on studying the role of residues/regions of the receptors for the calcitonin family of peptides. The calcitonin family of peptides and their receptors have broad pharmaceutical potential and have been implicated in the treatments of a variety of diseases including diabetes, migraine and osteoporosis. For most members of the calcitonin family peptides, their receptors have unique compositions comprising a GPCR (CLR or CTR) and a RAMP. The RAMP is an important component, not only to provide appropriate structure for ligands to bind but also to contribute to both receptor trafficking and function. Different RAMP/CLR or RAMP/CTR combinations show different pharmacologies. In order to generate drugs that target specifically to these receptors, a clearer understanding of the role of each component of the receptor in peptide binding is needed. Therefore this thesis is divided into two major studies. In the first study, mutagenesis (chimaera and SDM) has been carried out in RAMPs, aiming to identify the key residues/regions in RAMPs 1 and 3 that contribute to the interactions with ligand and receptor. The second study in this thesis investigated the role of residues in CTR by characterising a naturally occurring variant of hCT(a), i.e. Δ(1-47)hCT(a). These studies have generated novel findings and suggest some regions/residues that may play a role in the ligand to receptor interactions. Briefly, a single residue of RAMP3 (E74) has been demonstrated to be crucial for high affinity binding and high potency response of AM at the AM2 receptor. This is the first time that a single residue has been shown to be selectively involved in a specific peptide binding. In addition, for the first time, Δ(1-47)hCT(a) has been shown to be able to form the AMY1(a) receptor phenotype with RAMP1 despite lacking residues 1-47 at the N terminus. In addition, a novel role of RAMP has been reported. RAMP1 presence seemed to fully restore the reduced rAmy and CGRP potencies observed at this human CT(a) variant. This type of rescue role has never been described before for RAMPs.

8.2 Mutagenesis study in RAMPs 1 and 3

As RAMP1 and RAMP3 generate distinct receptor phenotypes with either CLR or CT(a), it was hypothesised that the different sequences possessed by RAMPs 1 and 3 (Chapter 4, Figure 4.1) contribute to the receptor specific pharmacology displayed by either CGRP vs
AM2 receptors or AMY1(a) vs AMY3(a) receptors. Two approaches were taken to identify residues or regions that are important for these pharmacological differences; chimaeras and strategic substitutions.

### 8.2.1 RAMP1/3 chimaera study

This study was carried out alongside another study using large chimaeras made by swapping helix 1, helices 1 and 2 and the entire N-terminal domains between RAMPs 1 and 3 (Qi et al., 2010). The data generated from the study using the large chimaeras suggested some contribution of the RAMP sequence to receptor pharmacology, however, it did not provide much information on which regions might be involved. To further pinpoint the specific regions in RAMP1 that may contribute to this receptor specific pharmacology, a series of N-terminal RAMP1 chimaeric mutants with every four consecutive residues replaced by the corresponding regions from RAMP3 were generated. Blocks of four residues were chosen as they broadly represent one turn in the helical structure of the N terminus of RAMPs. These chimaeras were characterised with both CLR (by a collaborating group in Aston University, UK, (Qi et al., 2010) and CT(a) in this thesis.

In total, 26 RAMP1/3 chimaeras have been functionally characterised with CT(a) for Tyr^haCGRP and hβCGRP responses; rAmy responses were also determined for some selected chimaeras. A previous study showed that amongst the cognate peptides for the calcitonin peptide receptors and some modified peptide analogues, Tyr^haCGRP and hβCGRP were the most selective at the RAMP1-based AMY1(a) receptor over the RAMP3-based AMY3(a) receptor (Hay et al., 2005). Both Tyr^haCGRP and hβCGRP activated AMY1(a) more potently than AMY3(a), displaying ~30-fold higher potency (Hay et al., 2005). On the other hand, rAmy is equally potent at both AMY1(a) and AMY3(a). Therefore based on the hypothesis, introductions of RAMP3 residues into AMY1(a) receptors would reduce Tyr^haCGRP and hβCGRP potencies whereas Amy potency would not be affected. In the chimaera experiments (Chapter 3), Tyr^haCGRP and hβCGRP responses at the WT AMY1(a) and AMY3(a) receptors were broadly consistent with published data, displaying higher potency at AMY1(a) than at AMY3(a) (Hay et al., 2005). However, they did not appear to be as selective as reported in the earlier study, only displaying ~7-fold and ~9-fold difference in Tyr^haCGRP and hβCGRP potencies, respectively.

The Tyr^haCGRP and hβCGRP responses generally agreed with each other, and most chimaeras that affected Tyr^haCGRP potency also altered hβCGRP potency. However, the
magnitudes of reduction were larger for Tyr°hαCGRP with the chimaeras in helix 1, loop 1 and helix 2 of RAMP1. This is in contrast to the chimaeras at the end of loop 2 and beginning of helix 3, whereas substantially larger effect in potency was seen with the chimaeras at region 86-93 for hβCGRP compared to Tyr°huCGRP (Chapter 3, Table 3.2). This suggests that there may be some difference in the binding pockets for Tyr°hαCGRP and hβCGRP in AMY$_{1(a)}$; the beginning of helix 3 might be more important for hβCGRP potency. This speculation is consistent with the data observed with the differential response seen with α- and βCGRP at the RAMP$_{86-89}$/CLR complex, which suggested some difference in the binding pockets for these peptides in the CGRP receptor (Section 8.2.3.1). In addition, that less effect was observed with the chimaeras near the TM domain than those at the N-terminal portion confirms the importance of the N-terminal domain in determining receptor pharmacology.

The data with chimaeras have shown that the association of RAMP1 with CT$_{(a)}$ is very sensitive to the amino acid changes as peptide potency was reduced at most mutant receptors containing the N-terminal RAMP1/3 chimaeras. These reductions cannot be fully explained by the functional effect induced by the amino acid substitutions. In the current study, Tyr°hαCGRP and hβCGRP both displayed less than 10-fold higher potency at AMY$_{1(a)}$ than AMY$_{3(a)}$. If the introduction of RAMP3 residues into RAMP1 mainly affected receptor function either by altering ligand binding or receptor activation but introduced little structural changes, a reduction of less than 10-fold in peptide potency would have been expected. However, most chimaeric receptors displayed much greater than 10-fold reduction in peptide potency (Chapter 3, Table 3.2). Therefore, the replacement of RAMP1 residues with those of RAMP3 may have introduced some general distortions to the protein structure which have disturbed its association with CT$_{(a)}$ and thus resulted in a depressed expression of AMY$_{1(a)}$ receptor phenotype at the cell surface.

As CT$_{(a)}$ can be expressed at the cell surface with or without RAMP and these RAMP1/3 chimaeras were not tagged, a direct measurement of AMY$_{1(a)}$ receptor expression at the cell surface was not possible in this study. The association of the chimaeras with CLR was instead measured to provide an estimation of the ability of these chimaeras to complex with a GPCR. However, the chimaeras apparently produced much less effect on cell-surface expression of the RAMP1/CLR complex than expected for the RAMP1/CT$_{(a)}$ complex, as only 8 of 26 N-terminal RAMP1/3 chimaeras showed significantly reduced receptor expression at the cell surface. With the single amino acid substitutions in RAMP1, the receptor cell-surface expression was more affected for the RAMP1/CT$_{(a)}$ complexes than the RAMP1/CLR
complexes even though the receptors contained the same mutations (Chapter 4). Therefore, it is likely that cell surface expression of RAMP1/CT(a) complexes containing the chimaeras was affected, although this could not be directly measured. In addition, the chimaeras had much smaller functional effects with CLR. Only six chimaeras caused significant reduction in CGRP potency; five of them were accompanied with significantly reduced receptor cell-surface expression (Qi et al., 2010). The agreement between the function and expression data observed with CLR further supports the speculation that the chimaeras had greater effect on the receptor expression with CT(a) than that indicated by the CLR expression data. It seems that the interaction between RAMP1 and CT(a) may be less robust compared to the RAMP1/CLR complex. A possible explanation is that there might be more contact points involved in RAMP1 to CT(a) association than with CLR, thus more effects were observed with residue substitutions. Structures of the protein complexes are needed to confirm this.

rAmy response was also determined at six selected RAMP1/3 chimaeras. rAmy is equally potent at AMY1(a) and AMY3(a) receptors, therefore it was hypothesised that the chimaeras would not affect rAmy potency at AMY1(a), unless the mutation had introduced substantial structural change and altered the receptor expression at the cell surface. RAMP350-53 and RAMP386-89 which induced significant reductions in response to the CGRPs also had reduced rAmy potency. However, the other four mutants did not introduce any significant change to rAmy potency although RAMP394-97 AMY1(a) showed ~10-fold reduction in TyrºhαCGRP and hβCGRP potency. It is possible that small changes observed in peptide potency (~10 or < 10) are more likely to reflect genuine changes in receptor function, whereas larger changes seen in peptide potency might have resulted from impaired CT(a) association that led to reduced receptor expression at the cell surface. In addition, the differential effects observed with rAmy and the CGRPs potencies suggest that there may be subtle differences in the binding pockets for these two peptides.

The fact that a large number of chimaeras had significant effects in peptide potency is rather surprising considering the high sequence homology and structural resemblance between RAMPs 1 and 3. Nevertheless, there are some differences observed in the RAMP3 model compared to the RAMP1 crystal structure of the N-terminal domains (Qi et al., 2010). Helix 1 in RAMP3 is more unwound and loop 2 and the beginning of helix 3 is less exposed in RAMP3 compared to RAMP1. In addition, the orientation of the C-terminal portion of RAMP3 was significantly different between RAMPs 1 and 3. These structural differences may have caused alterations in ligand binding and/or receptor activation when RAMP3
residues were introduced to RAMP1. It is apparent that the impact is generally larger for CT\((a)\) association than CLR, however, there are some patterns that have emerged from the data, providing some very valuable insight to structure-function relationship of RAMP1 in complex with CLR and CT\((a)\). The most striking difference is the role of helix 1. Helix 1 has been suggested to play little role in the CLR interactions or receptor function (Kusano \textit{et al.}, 2008). Its presence as a whole appeared to be necessary for maintaining the overall protein structure as the large chimaeras generated by exchanging helix 1 between RAMPs 1 and 3 showed either reduced or no association with CLR or CT\((a)\) (Qi \textit{et al.}, 2010). Amongst all the site-direct mutagenesis performed in helix 1, only one mutant M48A CGRP showed reduced cell-surface expression but CGRP and AM potencies were not affected (Simms \textit{et al.}, 2009). The chimaera data with CLR is consistent with the suggestion that helix 1 has a minor role in the interactions with peptide or CLR as all the chimaeras generated in this helix except RAMP338-41 did not significantly affect peptide potency or receptor expression at the cell surface (Figure 8.1b). On the other hand, helix 1 may have quite a significant role in RAMP1 association with CT\((a)\). As shown in Figure 8.1a, most chimaeras generated in helix 1 affected peptide potency when coexpressed with CT\((a)\), with larger effects observed at the N-terminal portion. Another region where large effects were seen with CT\((a)\) is at the neighbouring N-terminal portion of helix 3. Therefore, it is possible that the CT\((a)\)-interacting region in RAMP1 is located at the N-terminal parts of helices 1 and 3. This is in contrast with CLR-interacting region suggested by the chimaera data which locates at the diagonal stripe across the C-terminal part of helix 2 to the N-terminal part of helix 3 (Qi \textit{et al.}, 2010) (Figure 8.1b).

On the other hand, it is consistent between the chimaera data with CLR and CT\((a)\) that helices 2 and 3 are the major contributors to the receptor function. The chimaeras that affected peptide potency with CLR (Figure 8.1b) and those that showed a small effect in peptide potency with CT\((a)\) (Figure 8.1a) are mostly located on helices 2 and 3.
Figure 8.1 The crystal structure of the RAMP1 N-terminal domain (Kusano et al., 2008) showing the functional effects induced by RAMP1/3 chimaeras when complexed with (a) CT (a) and (b) CLR (Qi et al., 2010). Regions identified by the chimaeras that showed >100 fold (red), 10-100 fold (green) and ~10 or <10 fold reduction (yellow) in potency for Tyr°hαCGRP and hβCGRP are highlighted on the structure. Stick forms are shown for the chimaeras that reduced receptor expression at the cell surface when coexpressed with CLR. The backbone structure is displayed in blue.

8.2.2 Evaluation of chimaera approach

Given the long extracellular domains of the receptor components and large endogenous peptide ligands, it is likely that multiple points of contact exist in the interactions between the calcitonin family of peptides and their receptors. Therefore, as an initial screen of the regions of the RAMP that are possibly involved in ligand to receptor interactions, large chimaeras exchanging the major helices between RAMPs 1 and 3 (Qi et al., 2010) and a series of small chimaeras with every four consecutive residues in RAMP1 replaced by the corresponding residues from RAMP3 were characterised. Nevertheless, a large number of chimaeras induced large changes to peptide potency which has made it difficult to interpret the data. The tendency for chimaeric mutants to generate large effects has been observed in other studies (Kuwasaeko et al., 2001). In contrast, amino acid substitutions seemed more likely to produce
more specific effects which are not induced by large structural distortions. Amino acid substitution approach has been shown to be useful to study the role of residues in RAMP1 in complex with CT\textsubscript{(a)} (Gingell \textit{et al.}, 2010). Therefore, strategic substitution with individual amino acid mutations introduced to RAMPs 1 and 3 was employed in the subsequent study in this thesis.

8.2.3 Strategic substitutions in RAMPs 1 and 3

In this study, it was hypothesised that residues conserved between RAMPs 2 and 3 (forming the AM receptors with CLR or AMY\textsubscript{2(a)} and AMY\textsubscript{3(a)} receptors with CT\textsubscript{(a)}) but that are different in RAMP1 (forming the CGRP receptor with CLR or AMY\textsubscript{1(a)} receptors with CT\textsubscript{(a)}) are responsible for defining the pharmacology of these receptors (sequence alignment refers to Chapter 4, Figure 4.1). Eight such residues were identified in the sequence alignment and reciprocal RAMP1 and RAMP3 mutants with residues swapped at these positions were generated and characterised with both CLR and CT\textsubscript{(a)}.

8.2.3.1 RAMPs 1 and 3 mutants with CLR

The most significant finding was from the amino acid substitutions at position 74 of RAMPs 1 and 3. A previous study has suggested that E74 of RAMP3 was important for AM potency (Hay \textit{et al.}, 2006a). Consistent with this published data, the E74W mutant in RAMP3 made in this thesis selectively reduced AM potency at the AM\textsubscript{2} receptor, but CGRP potency was not affected (Chapter 5). An opposite effect with a similar magnitude of change in AM potency was observed with the reciprocal RAMP1 mutant W74E in the CGRP receptor, but CGRP potency was not altered (Chapter 4). Both mutations did not significantly affect receptor expression at the cell surface. These data have demonstrated the important contribution of E74 specifically to AM potency. Additional characterisation with another form of hAM, AM\textsubscript{15-52}, has eliminated a role for the N-terminal 14 amino acids in the AM interactions with or near E74 of RAMP3 (Chapter 6). A recent publication has further pinpointed the E74-interacting region in AM to the last 9 amino acids at its C terminus (Robinson \textit{et al.}, 2009). This is consistent with the two-domain binding mechanism suggested for the family B GPCRs where the C terminus of the peptide binds to the N terminus of the receptor to induce an affinity trap in step 1 (Hoare, 2005).

Another rather striking observation was with AM2 (47 amino acids) (Chapter 6). AM2 is produced from a different gene to AM and shares only limited sequence identity with AM
Chapter 8

(Refer to Chapter 1 Figure 1.1). Very interestingly, as with AM, residue swapping at position 74 between RAMPs 1 and 3 also induced a similar reciprocal effect on AM2 potency (Chapter 6). This suggests that E74 is important for both AM and AM2 interactions with the AM2 receptor. Therefore, the potential residue involved in the interaction at or near position 74 in RAMP3 may reside at the conserved regions/residues between AM and AM2. Two hot spots of these conserved residues have been identified in the amino acid sequence alignment (Refer to Chapter 1, Figure 1.1), the N-terminal residues 5-26 and the C-terminal 37-47 in AM2. Based on the study performed by Robinson et al. (Robinson et al., 2009) and the two-domain binding mechanism proposed for the family B GPCRs (Hoare, 2005), it is more likely that the conserved residues within the C-terminal 37-47 region may be involved in the interactions at or near position 74 of RAMP3. In addition, AM2 appeared to behave differently in the current study from some previous publications in terms of its pharmacological selectivity. In the characterisation performed in this thesis, AM2 displayed an equivalent potency at the AM2 receptor compared to AM and it was more selective at the AM2 receptor over the CGRP receptor by approximately 10-fold. However, AM2 was found to be less potent than AM at the AM2 receptor (Roh et al., 2004) and was non-selective at the AM2 and CGRP receptors (Hay et al., 2005; Roh et al., 2004). The study carried by Roh et al. was performed in HEK293T cells as opposed to Cos 7 cells used in this thesis; the different cellular background may be a possible explanation. However, the reason for these different observations with AM2 is not clear with the study performed by Hay et al. where Cos 7 cells were also used. The data indicate that the nature of AM2 interactions with the receptors is certainly more complex than it appeared to be in the initial discovery and characterisation of this peptide. Furthermore, the magnitudes of change in AM2 potency observed at the E74W AM2 and the W74E CGRP receptors were different (~2-fold vs ~5-fold), emphasising that AM2 may have a different mode of interaction with the two receptors. 

In addition, for the first time the crucial role of E74 of RAMP3 in AM binding has been shown in this thesis. E74W mutation in RAMP3 led to reductions in both $^{125}$IAM$_{13-52}$ affinity and maximum specific binding whereas opposite effects were seen with the W74E mutation in RAMP1 when they were coexpressed with CLR. The importance of E74 is also revealed in the amino acid sequence alignment of the RAMPs across different species which shows that E74 is highly conserved (Qi et al., 2008, supplement).

As position 74 of the RAMP appeared to be important to AM interaction, further amino acid substitutions were carried out in both RAMP 1 (Glu, Phe, Tyr, Ala and Asn) and RAMP3
(Trp, Phe, Tyr, Ala, Ser, Thr and Asn) with CLR to elucidate the nature of the interaction at this position (Chapter 6). All the substitutions introduced to both RAMPs 1 and 3 had no significant effect on receptor expression at the cell surface. There was a generalised reduction in AM potency for the RAMP3 mutants but enhancement in AM potency for the RAMP1 mutants, emphasising the crucial role of the native E74 in RAMP3 to AM potency. Nevertheless, Phe and Tyr substitutions generated some intriguing observations (Chapter 6). Whilst Phe but not Tyr substitution in RAMP3 significantly reduced AM potency at the AM2 receptor, Phe did not affect AM potency but Tyr significantly enhanced AM potency at the CGRP receptor when they were substituted into RAMP1. The fact that Tyr is identical to Phe apart from an extra hydroxyl group present on the aromatic ring in Tyr suggests that the hydroxyl group may have restored an important interaction that was interrupted by the presence of the bulky aromatic side chain. This rescue may have resulted from a potential hydrogen bond formation with the hydroxyl group in Tyr, which may also be formed at the native E74. Nevertheless, this interaction is not as strong as that formed by the native E74, possibly due to the presence of the bulky aromatic ring in Tyr. This may explain the slightly smaller effect on AM potency observed with Tyr substitution compared to Glu in both RAMPs 1 and 3.

Further substitutions with small amino acids Ser and Thr containing a hydroxyl group and Ala which does not have any functional group have suggested that the size and geometry may be important for the bond formation at position 74. In addition, substitutions with residues of similar size to Glu but with either a polar group (Asn) or opposite charge (Arg) emphasised the crucial role of the negative charge in the native Glu. These data are consistent with another study where the Lys (positively charged) and Gln (polar) substitutions led to reduced AM potency at the AM2 receptor, whilst Asp which is a very similar amino acid to Glu also with a negative charged carboxyl group did not significantly affect AM potency (Hay et al., 2006a).

The nature of the interaction at position 74 in RAMP3 can be speculated based on the data generated in this study. First, the size of the residue at this position is important; small or bulky residues will reduce AM potency. Amino acids of appropriate size and geometry are needed to enable bond formation at position 74. Secondly, a salt bridge containing an electrostatic bond and hydrogen bond might be formed at position 74; this requires the residue to be negatively charged to strengthen the bond formation. However, whether E74 is directly involved in the AM interactions with the receptor by forming part of the binding
pocket or indirectly through introducing conformation changes to the AM binding pocket remains unclear. It is possible E74 has an indirect role as only small effects were observed in the amino acid substitutions performed at this position.

In contrast to the crucial role of E74 of RAMP3 to high potency AM response at the AM$_2$ receptor, W74 of RAMP1 may not play a similar role in the CGRP receptor. In fact, the presence of the bulky Trp at position 74 seemed to cause a steric hindrance which reduced AM potency. It is possible that W74 may contribute to the less potent response of AM observed at the CGRP receptor.

On the other hand, amino acid substitutions introduced at position 74 in RAMPs 1 and 3 generally produced little effect on CGRP response (Chapter 6). This general lack of effect is consistent with earlier studies where Asp, Gln and Lys substitutions in RAMP3 and Ala and Lys substitutions in RAMP1 at position 74 all showed WT-like CGRP response in the AM$_2$ or CGRP receptor, respectively (Hay et al., 2006a; Moore et al., 2010; Qi et al., 2008). Despite that W74 has been identified as one of the CGRP-interacting residues on the crystal structure of the RAMP1 N terminus (Kusano et al., 2008), mutagenesis data generated in this study and other studies (Hay et al., 2006a; Qi et al., 2008) together show that position 74 in RAMPs is unlikely to be involved in the CGRP to receptor interactions. Nevertheless, W74 has been demonstrated in a few studies to potentially interact with small molecule CGRP antagonists olcegepant and telcagepant (Hay et al., 2006a; Koth et al., 2010; Mallee et al., 2002; Moore et al., 2010).

A second residue that was identified in the amino acid substitution study was F93 of RAMP1 (Chapter 4). In the current study, F93I CGRP receptor led to reduced h$\alpha$CGRP potency in the absence of any change in cell-surface expression, suggesting that it may play a role in the interaction with h$\alpha$CGRP. However, the effect seen at position 93 seems to be substitution dependent. F93A RAMP1/CLR showed significantly reduced cell-surface expression (Simms et al., 2009) and reduced total CGRP binding (Kuwasaki et al., 2003a) in the absence of a change in potency. The latter data indicates that F93 is important for CLR interaction which is consistent with the speculation made from the crystal structure of RAMP1 N-terminal domain (Kusano et al., 2008). The effect seen with Ile substitution in this study may be a substitution specific phenomenon. It is possible that the bulkier F93I substitution allowed maintenance of the CLR/RAMP interaction but had a smaller conformational effect on the receptor complex leading to a small change in CGRP binding or receptor activation. Further
investigation is needed to determine the importance of this residue. Furthermore, the A89V RAMP3 mutant also displayed interesting behaviour with an increase in hAM potency in the absence of any change in CGRP potency or expression of the receptor complex (Chapter 5). However, no change in hAM or hαCGRP potency was observed with its reciprocal RAMP1 mutant V89A (Chapter 4). This may suggest a differential interaction environment at position 89 in RAMPs 1 and 3.

Besides the mutations that have been studied in this thesis, there have been many mutagenesis studies performed in the RAMPs aiming to identify the residues that are important for ligand binding or receptor association. It is important to map the effects of these mutations on the newly available RAMP1 structure and RAMP3 model. Figure 8.2 shows the SDM data generated in other studies and in this thesis. Although the mutagenesis data are considerably less for RAMP3 than RAMP1, they both agree that residues important for ligand and receptor recognition mainly reside on helices 2 and 3. These assignations of the residues and their effects to the RAMP structure/model can provide guidance for further mutagenesis work.
Figure 8.2 (a) the crystal structure of the human RAMP1 N terminus (Kusano et al., 2008) and (b) the structural model of the human RAMP3 N terminus (Bailey et al., 2010) showing the residues upon which mutagenesis has been performed in the literature (Bailey et al., 2010; Hay et al., 2006a; Kuwasako et al., 2003a; Moore et al., 2010; Simms et al., 2009; Simms et al., 2006) and this thesis. Differential colouring reflects the effects of amino acid substitutions at residues that have been characterised for either CGRP responses at the CGRP receptor (a) or AM responses at the AM2 receptor (b). Green, peptide potency was not affected; red, peptide potency was reduced by <100 fold; purple, peptide potency was reduced by >100 fold; yellow, peptide potency was enhanced by <100 fold. In addition, residues shown in stick form indicate that the mutations also reduced cell-surface expression of the receptor complex. Blue (a) or cyan (b) represent the residues which have not been characterised by point mutation in RAMP1 and RAMP3, respectively.

The data in this thesis have also suggested some additional residues of importance for both CLR and ligand interactions on helix 3. The beginning of helix 3 at the junction with the connecting loop to helix 2 appears to be an important region for peptide recognition; residue swapping between RAMP 1 (Chapter 4) and RAMP3 (Chapter 5) at this region (86-89)
reveals some very interesting pharmacology. The RAMP1 chimaera (RAMP3\textsubscript{86-89}) induced a reduction in hαCGRP and hAM potency when coexpressed with CLR. This was in the absence of any significant change in cell-surface expression of the receptor, suggesting the importance of this region for the interactions with these two peptides. Individual amino acid substitutions with RAMP3 residues introduced to RAMP1 at the three non-conserved residues (87, 88 and 89) in this region did not produce enough distortion to the peptide binding to induce any change to either peptide response. This may suggest that the interaction needs multiple contact points or the residues in this region act together to provide the appropriate structural support for peptide binding. Very interestingly, data generated from hβCGRP response suggested that region 86-89 in RAMP1 does not have a role in hβCGRP recognition. There are only three residues that are different between hαCGRP and hβCGRP, at positions 3, 22 and 25. The N-terminal residue 3 is most likely to be involved in the interaction with the J-domain of the receptor, based on two-domain binding mechanism suggested for the family B GPCRs (Hoare, 2005); therefore residues at position 22 and 25 are more likely to be responsible for the differential peptide responses observed; they may be in proximity to region 86-89 of RAMP1 when bound. On the other hand, the characterisation with the reciprocal mutant RAMP1\textsubscript{86-89} have eliminated the involvement of region 86-89 to any of the three peptide responses in RAMP3. This data has provided experimental support to the RAMP3 model (Bailey \textit{et al.}, 2010; Qi \textit{et al.}, 2010) where the loop by which region 86-89 sits next to is positioned differently compared to the corresponding region in RAMP1 (Figure 8.3). The importance of this flexible loop of RAMP1 has also been demonstrated by amino acid substitutions of the residues in this region. Alanine substitutions at W84, P85, N86 in RAMP1 all reduced hαCGRP potency at the CGRP receptor accompanied with a reduced receptor expression at the cell surface (Moore \textit{et al.}, 2010; Simms \textit{et al.}, 2009). In addition, W84 has also been suggested to play an important role in small molecule CGRP antagonist telcagepant and an analogue of olcegepant interactions (Moore \textit{et al.}, 2010). This loop may also be important for either peptide interaction or CT\textsubscript{(a)} interaction in the AMY\textsubscript{1(a)} receptor. This speculation is supported by the chimaera RAMP\textsubscript{86-89} data with large reductions (>100-fold) in both Tyr\textsuperscript{α}hαCGRP and hβCGRP potency at the AMY\textsubscript{1(a)} receptor in the current study and the W84A and W84F mutations with reduced rAmy and hαCGRP potency accompanied with reductions in the cell-surface expression of the AMY\textsubscript{1(a)} receptor in another study (Gingell \textit{et al.}, 2010).
8.2.3.2 RAMPs 1 and 3 mutants with CT$_{(a)}$

In contrast to CLR, more effects were seen with the mutations introduced into either RAMP1 (Chapter 4) or RAMP3 (Chapter 5) when they were coexpressed with CT$_{(a)}$. In the substitutions in RAMP1, five out of eight mutations significantly altered peptide potency for either one of both peptides (rAmy and hβCGRP), these were W74E, E88L, V89A, F93I and S103N. These effects in receptor function were likely caused by some structural distortions to the RAMP1 to CT$_{(a)}$ association sites as all these mutations also induced significant alterations to the receptor expression at the cell surface. These data are consistent with the chimaera data, further emphasising that the RAMP1/CT$_{(a)}$ complex is more labile to residue changes compared to the RAMP/CLR complex.

The RAMP3 reciprocal mutants generated less effect than the RAMP1 mutants with CT$_{(a)}$. Three mutants including E74W, I93F and N103S caused significant reductions in both rAmy and hβCGRP potency. As explained in Chapter 5, a direct measurement of the receptor cell-surface expression was not possible. Nevertheless, the observation that all the RAMP1 mutants (with CT$_{(a)}$) that affected peptide potency was paralleled by altered receptor expression at the cell surface may suggest that the reduced peptide potency seen with the three RAMP3 mutants might have been caused by the potentially reduced receptor cell-surface expression.
As mentioned above, mutations E74W RAMP3 and W74E RAMP1 induced a reciprocal effect in hAM potency when analysed with CLR, it is interesting that these two mutations also affected rAmy potency in a similar manner when coexpressed with CT(a). Unlike with CLR, significantly altered receptor expression was also observed for the RAMP1 mutant. The data may suggest a potential involvement of the residues at position 74 in RAMPs 1 and 3, either in the interactions with rAmy or CT(a). In contrast, W74K and W74A mutations in RAMP1 did not affect rAmy potency (Hay et al., 2006a). Further investigation is needed to determine the role of this residue in the Amy receptors. Another mutation F93I RAMP1, which selectively reduced hαCGRP potency in the CGRP receptor, also induced significant reductions in rAmy and hβCGRP potencies in the AMY1(a) receptors but accompanied with a reduced receptor expression a the cell surface.

8.3 Δ(1-47)hCT(a) characterisation

Due to the importance of the N terminus of the receptor as well as the RAMP in ligand interactions, this thesis also aimed to characterise a naturally occurring variant of the human calcitonin receptor Δ(1-47)hCT(a) with and without RAMP1 (Chapter 7). The main hypothesis explored was that the N-terminal truncation in Δ(1-47)hCT(a) impaired its ability to associate with RAMP1 and thus reduced the AMY1(a) receptor phenotype. This hypothesis was made based on a previous study using chimaeras between CLR and PTH-1 (parathyroid hormone receptor 1) which identified that residues 23-60 in the N terminus of mouse CLR were required and sufficient for its association with mouse RAMP1 (Ittner et al., 2005). For the first time, the data from this study have shown that despite lacking the first 47 residues in the N terminus, Δ(1-47)hCT(a) is still capable of associating with RAMP1 to constitute an AMY1(a) receptor phenotype. However, the expression of Δ(1-47)hAMY1(a) was reduced by ~40% compared to the full length hAMY1(a). Nevertheless, Δ(1-47)hAMY1(a) appeared to be fully functional at the cell surface, displaying equivalent peptide responses to the full length receptor for all agonists tested except for hCT. The peptide potency order was sCT ≈ hCT > rAmy > CGRP for AMY1(a) and sCT > rAmy > CGRP > hCT for Δ(1-47)hAMY1(a). The reduced hCT potency may be explained by the removal of residue T30 in Δ(1-47)hCT(a), which has been demonstrated to interact with residue 26 in hCT (Dong et al., 2004a).

Further analysis of receptor cell-surface expression measuring the hCT(a) expression using an antibody binding to the N terminus of hCT(a) beyond residues 47 showed that Δ(1-47)hCT(a) expression was not reduced by the N-terminal 1-47 truncation both alone or in the presence...
of RAMP1. Therefore, the reduced $\Delta(1-47)hAMY_{1(a)}$ expression measured by mycRAMP1 expression at the cell surface was not caused by the reduced hCT$_{(a)}$ expression in this complex, but by an impaired RAMP1 to hCT$_{(a)}$ association. This suggested that region 1-47 of hCT$_{(a)}$ was important to its association with RAMP1, however, there may be some other association points beyond this region. The region important for RAMP1 association with CLR has been suggested to be the N-terminal 23-60 in a previous study (Ittner et al., 2005). Based on the reduced RAMP1 association seen with $\Delta(1-47)hCT_{(a)}$ in this thesis, it is possible the corresponding region in hCT$_{(a)}$ plays a similar role; further mutagenesis on residues further away from 47 can potentially provide evidence for this speculation.

There was a generalised reduction in peptide potency for the agonist responses at $\Delta(1-47)hCT_{(a)}$ compared to the full length hCT$_{(a)}$. These reductions did not result from depressed cell-surface expression as $\Delta(1-47)hCT_{(a)}$ had equivalent expression to hCT$_{(a)}$, suggesting that there may be one or more interaction sites within the first 47 amino acids of hCT$_{(a)}$ that are important for these peptide potencies, or the truncation has introduced some changes to receptor conformation which affected the peptide to receptor interactions. Very interestingly, peptide potencies for rAmy and three forms of CGRP were restored by the RAMP1 association with $\Delta(1-47)hCT_{(a)}$, displaying equivalent or higher potencies for these peptides to hAMY$_{1(a)}$. This may suggest that RAMP1 has a role in stabilising these ligand to receptor interactions either by directly providing some binding sites for these ligands or changing hCT$_{(a)}$ conformation upon association to allow high potency response of rAmy and CGRP. The data generated in this part of thesis serve as the first piece of evidence for this novel role of RAMP1 to the receptor function.

In contrast, peptide potency for hCT was not rescued by the presence of RAMP1; a significant reduction in potency was seen at $\Delta(1-47)hAMY_{1(a)}$ compared to hAMY$_{1(a)}$. The presence of RAMP1 did not seem to affect hCT potency in the assays performed in this chapter as hCT was equally potent at the full length hCT$_{(a)}$ and AMY$_{1(a)}$. RAMP1 association with hCT$_{(a)}$ may have little impact to the hCT interaction with the receptor. The unaffected hCT response by RAMP1 was different from rAmy and CGRP, suggesting that the binding pocket of hCT might be different to those of rAmy and CGRP on hCT$_{(a)}$ and hAMY$_{1(a)}$. 

224
8.4 Conclusion and future work

In this thesis a large amount of mutagenesis data have been generated in RAMPs 1 and 3 that covers the entire N terminus. Structure-function relationships have been determined for these RAMPs 1 and 3 residues in complex with CLR or CT_{(a)}. These data have provided some valuable insights to structure-function relationships of regions/residues in the N terminus of RAMP. In particular, unlike CLR, which is much better characterised by the RAMP mutagenesis data available in the literature, knowledge of structure-function relationship of RAMP with CT_{(a)} is severely lacking. Therefore, the data generated in this thesis have greatly contributed to our understanding of the role of RAMP in the interactions with CT_{(a)}. Patterns have emerged from the data generated, identifying the roles of different regions in the N terminus of RAMP1. Despite the high sequence homology between CLR and CT_{(a)}, their interactions with RAMP1 seem different. The interaction between CT_{(a)} and RAMP1 may be intrinsically weaker than in the CLR/RAMP complex and different regions in the RAMP1 N terminus appeared to be involved in the association with CLR and CT_{(a)}.

Residues that are important for some specific ligand to receptor interactions have been identified in this thesis. The crucial role of E74 in RAMP3 for AM interactions with the receptor has been demonstrated. This is the first time that a single residue has been conclusively assigned to have a role in a specific peptide binding and response for the calcitonin peptide family. The data has also shown that F93 and V89 of RAMP1 may be important for CGRP and AM potency at the CGRP receptor, respectively. In addition, region 86-89 of RAMP1 appeared to be involved in both CGRP and AM interactions. These data generated in this thesis have suggested the importance of these residues, however, it is still an open question as to whether these RAMP residues/regions modulate receptor pharmacology by directly providing points of contact or allosterically altering CLR conformation to allow selective ligand binding. Therefore, other approaches including photoaffinity labelling (Dong et al., 2009; Dong et al., 2010; Dong et al., 2004a, 2004b; Pham et al., 2005; Pham et al., 2004), bioluminescence resonance energy transfer (Harikumar et al., 2009; Héroux et al., 2007) and NMR chemical shift analysis (Moore et al., 2010) could be performed targeting the residues identified in this thesis. These are more direct approaches exploring spatial approximations between distinct residues within the ligand and receptor. In particular, direct or indirect contacts of the residues that are potentially involved in ligand to receptor interactions can be speculated using these approaches. Determinations from these
experiments could potentially provide valuable information and targets for rational drug design.

In chapter 4 and 6, specific regions and residues were suggested in the peptide ligand that may be involved in interactions with RAMP1; region 86-89 of RAMP1 with either residue 22 or 25 of hnCGRP and residue 74 of RAMP3 with region 37-47 of AM2. In further studies, amino acid substitutions (mutagenesis) or photolabile residues (photoaffinity labelling) could be introduced into these positions in the peptides/ligands to pinpoint the residues that are involved. These experiments would provide additional evidence for determining the binding mechanism for the calcitonin family peptides.

In this thesis, a thorough pharmacological characterisation was carried out on a naturally occurring human calcitonin receptor variant, Δ(1-47)hCT(a). For the first time, it was shown that Δ(1-47)hCT(a) is able to interact with RAMP1 to form a functional AMY1(a) at the cell surface. However, expression analysis showed that RAMP1 association was impaired. The region important for RAMP1 association with CLR was suggested to be the N-terminal 23-60 (Ittner et al., 2005). The impaired RAMP1 association observed with the 1-47 truncation indicates that the corresponding region in hCT(a) may play a similar role. Further truncations from 47 could potentially identify the region in hCT(a) that is responsible for RAMP1 association. In addition, as Δ(1-47)hCT(a) has been only characterised with RAMP1 in this thesis, similar characterisation could be performed with RAMPs 2 and 3. In particular, expression data will allow the comparison across all three RAMPs, to answer the question whether they associate to the same region of hCT(a) to generate the hAMY(a) receptors. It would also be interesting to see whether the rescue of rAmy potency by RAMP1 also applies to RAMPs 2 and 3. Such information will contribute to our understanding of the RAMP modulation and peptide interactions in different the Amy receptor subtypes.

A biased agonist which selectively activates β-arrestin but not G protein mediated signalling has been reported for PTH1R (Gesty-Palmer et al., 2009). The binding of the biased agonist may lock the receptor in a particular conformation that changes its signalling. Likewise, mutations introduced to the receptor could have a similar effect; it may change the receptor conformation to enhance or reduce some receptor-mediated signalling events but not others. Therefore there is a possibility that the truncation in Δ(1-47)hCT(a) could bias signaling by producing a receptor with a different conformation. Studying other signalling events such as
measuring intracellular ERK1/2 phosphorylation may show some different effects from those observed in the cAMP assays.

Furthermore, for rational drug design, it is important for the structure of the calcitonin family receptors to be determined. The RAMP1 crystal structure and a RAMP3 model of the N-terminal domain have been published (Bailey et al., 2010; Kusano et al., 2008). This structural information is very helpful for interpreting the experimental data generated in this thesis. Some structural identification of RAMP2 is thus needed to help design studies investigating the structure-function relationship of the RAMP2 residues and interpret data generated. In addition, CLR and CTR structures are also needed. A recent study determined the NMR structure of the extracellular domains of the RAMP1/CLR complex (Koth et al., 2010). In this publication, the authors suggested that the crystal structure of this extracellular complex has also been resolved and about to be published. Similar structural determination is also needed for CTR. These structures will allow the accommodation of the residues identified in the mutagenesis study into the structure and thus provide more informed targets for further investigations that may help with rational drug design.

In conclusion, studies performed in this thesis have augmented our understanding of the structure-function relationship of the receptor components for the calcitonin family peptides and their binding mechanisms. Some residues and regions that may play important roles in the ligand to receptor interactions have been identified. They could potentially become targets for future investigations for rational drug design for treating diseases such as diabetes, bone disease and cancer.
The table below lists the primer sequences that were used for generating mutants in this thesis. F: forward primer; R: reverse primer.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Template</th>
<th>Primer sequence</th>
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<tr>
<td><strong>Chapter 3</strong></td>
<td></td>
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| RAMP3<sub>38-41</sub> | mycRAMP1 | F: GGTGCCCTCTCCGGCCGCTCTGCGGCAACCCAGTTCCAGGTA G  
R: CTACCTGGAACTGGTGCGCGAGCAGCCGGCGGAGGAGGCCACC  |
| RAMP3<sub>62-65</sub> | mycRAMP1 | F: GTGTGACTGGGCGAGGTTCATCGTGTA CAGGAGCTTGCC  
R: GGCCAGCTCCCCCTGTAGATACAGGAAACCTGCCCAGTCACAC  |
| RAMP3<sub>90-93</sub> | mycRAMP1 | F: CTCACCCAGTTCCAGGATGACATGGAGGCCGTCG  
R: CGACGGCCTCCATCGTCATCTCGAAGTGCAGTGC  |
| **Chapter 4** | | |
| A34E | mycRAMP1 | F: GGCTAAACTACGGTGAGCTCTCCCTCCGGAGGC  
R: GCTCCCGGAGGAGCTCACCGTATGAGC  |
| V46D | mycRAMP1 | F: CTCACCAGTTCCAGGATGACATGGAGGCCGTCG  
R: CGACGGCCTCCATCGTCATCTCGAAGTGCAGTGC  |
| W74E | mycRAMP1 | F: GGCCGACTGCACCGAGCACATGGCGGAGAAGC  
R: GCTTCTCCGCCCATGTCGCGGCAAGTGCAGTGC  |
| A87P | mycRAMP1 | F: GCTGCTTCTGGCCCAATCCAGAGGTTCATCCTGG  
R: GAACCTGTCCACCTCTGGATTGGGCCAGAAGC  |
| E88L | mycRAMP1 | F: GCTTCTGGCCCAATTCGACATGGGAGTTCTTCTGG  
R: CCAGGAAGAATCTGCTCCACCAATGCATTGGGCCAGAAGC  |
| V89A | mycRAMP1 | F: GGCCCAATGCAGAGGAGTGCACAGGTTCTTCTGG  
R: CCAGGAAGAATCTGCTCCACCAATGCATTGGGCCAGAAGC  |
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**Chapter 5**

**Chapter 6**

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<td>mycRAMP1</td>
<td>F: GCTGGCCCACTGCACTTCCACATGGGAGGAAGG</td>
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### Appendix

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### Chapter 7

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<td>F: CATCTGCCATCAGGAGCTGAGGAATGAAACCAGCC</td>
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<tr>
<td>Δ(1-47)hCT&lt;sub&gt;(a)&lt;/sub&gt;</td>
<td>Δ(1-47)hCT&lt;sub&gt;(a)-&lt;/sub&gt;I347</td>
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<td>R: GCTTCTCCGCACTGCACCTACCACATGGCGGAGAAGC</td>
</tr>
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</table>
Appendix

The original sequencing data are shown below to demonstrate the successful introductions of the mutations generated in this thesis.

mycRAMP1 RAMP$_{\text{38-41}}$; base changes: GAGCTCTGCCTC → CCGCTCTGCGGC

mycRAMP1 RAMP$_{\text{62-65}}$; base changes: ACCATCAGGAGC → TTCATCGTGTAC
Appendix

mycRAMP1 RAMP3\textsubscript{90.93}; base changes: GACAGGTTCCTC → CAGGGGTCATC

mycRAMP1 A34E; base changes: GCC → GAG
mycRAMP1 V46D; base changes: GTA → GAT

mycRAMP1 W74E; base changes: TGG → GAG
mycRAMP1 A87P; base changes: GCA → CCA

mycRAMP1 E88L; base changes: GAG → TTG
Appendix

mycRAMP1 V89A; base changes: GTG → GCG

mycRAMP1 F93I; base changes: TTC → ATC
Appendix

mycRAMP1 S103N; base changes: AGC → AAC

untagged RAMP3 E35A; base changes: GAG → GCG
untagged RAMP3 D46V; base changes: GAC → GTC

untagged RAMP3 E74W; base changes: GAG → TGG
untagged RAMP3 P87A; base changes: $CCC \rightarrow GCC$

untagged RAMP3 L88E; base changes: $CTG \rightarrow GAG$
untagged RAMP3 A89V; base changes: GCC → GTC

untagged RAMP3 I93F; base changes: ATC → TTC
untagged RAMP3 N103S; base changes: AAC → AGC

untagged RAMP3 RAMP186-89; base changes: CCCCTGGCC → GCCGAGGTC
Appendix

mycRAMP1 W74F; base changes: TGG → TTC

mycRAMP1 W74Y; base changes: TGG → TAC
Appendix

mycRAMP1 W74A; base changes: TGG → GCG

mycRAMP1 W74N; base changes: TGG → AAC
untagged RAMP3 E74F; base changes: GAG → TTC

untagged RAMP3 E74Y; base changes: GAG → TAC
untagged RAMP3 E74A; base changes: GAG → GCG

untagged RAMP3 E74S; base changes: GAG → TCG
untagged RAMP3 E74T; base changes: GAG → ACG

untagged hRAMP3 E74R; base changes: GAG → CGG
untagged hRAMP3 E74N; base changes: GAG → AAT

Δ(1-47)hCT<sub>(a)</sub>-I347 (mutation P447L); base changes: CCG → CTG (reverse primer was used for sequencing, thus the changes shown in the sequence trace below are CGG → CAG)
Δ(1–47)hCT<sub>(a)</sub> (mutation I347T); base changes: ATC → ACC (reverse primer was used for sequencing, thus the changes shown in the sequence trace below are GAT → GGT)
References


References


References


References


References


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References


References


