RESEARCH PAPER

Structure–activity relationships of the N-terminus of calcitonin gene-related peptide: key roles of alanine-5 and threonine-6 in receptor activation

Debbie L Hay1, Paul W R Harris2, Renata Kowalczyk2, Margaret A Brimble2, Dan L Rathbone3, James Barwell3, Alex C Conner4 and David R Poyner3

1School of Biological Sciences and Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Auckland, New Zealand, 2School of Chemical Sciences and Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Auckland, New Zealand, 3School of Life and Health Sciences, Aston University, Birmingham, UK, and 4Medical School, University of Birmingham, Birmingham, UK

BACKGROUND AND PURPOSE
The N-terminus of calcitonin gene-related peptide (CGRP) is important for receptor activation, especially the disulphide-bonded ring (residues 1–7). However, the roles of individual amino acids within this region have not been examined and so the molecular determinants of agonism are unknown. This study has examined the role of residues 1, 3–6 and 8–9, excluding Cys-2 and Cys-7.

EXPERIMENTAL APPROACH
CGRP derivatives were substituted with either cysteine or alanine; further residues were introduced at position 6. Their affinity was measured by radioligand binding and their efficacy by measuring cAMP production in SK-N-MC cells and β-arrestin 2 translocation in CHO-K1 cells at the CGRP receptor.

KEY RESULTS
Substitution of Ala-5 by cysteine reduced affinity 270-fold and reduced efficacy for production of cAMP in SK-N-MCs. Potency at β-arrestin translocation was reduced by ninefold. Substitution of Thr-6 by cysteine destroyed all measurable efficacy of both cAMP and β-arrestin responses; substitution with either alanine or serine impaired potency. Substitutions at positions 1, 4, 8 and 9 resulted in approximately 10-fold reductions in potency at both responses. Similar observations were made at a second CGRP-activated receptor, the AMY1(a) receptor.

CONCLUSIONS AND IMPLICATIONS
Ala-5 and Thr-6 are key determinants of agonist activity for CGRP. Ala-5 is also very important for receptor binding. Residues outside of the 1–7 ring also contribute to agonist activity.
**Introduction**

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide, which in humans and rodents is found in two forms: α and β. It is an abundant neuropeptide that is widely distributed throughout the sensory nervous system. It is an extremely potent vasodilator, which is involved in neurogenic inflammation. CGRP receptor antagonists reduce migraine pain in clinical trials (Recober and Russo, 2009). CGRP forms a family with calcitonin (CT), adrenomedullin (AM), AM 2/intermedin, amylin and calcitonin receptor-stimulating peptide (CRSP) (Takei *et al*., 2004; Katafuchi *et al*., 2009).

These peptides act on the CT or CT receptor-like receptors (CLR), which are family B GPCRs (receptor nomenclature follows Alexander *et al*., 2013). CLR in complex with the accessory protein receptor activity-modifying protein 1 (RAMP1) gives the CGRP receptor, whereas CLR with RAMP2 or 3 gives AM and AM receptors (receptor nomenclature follows Alexander *et al*., 2013; Supporting Information Table S1). The CT receptor complexes with RAMPs to give the AMY, AMY and AMY receptors for amylin (Mclatchie *et al*., 1998; Christopoulos *et al*., 1999; Muff *et al*., 1999); these exist as either (a) or (b) forms depending on the splice variant of CT receptor that is involved (Poyner *et al*., 2002). CGRP is a potent agonist at the AMY receptor (Hay *et al*., 2005; Bailey *et al*., 2012).

The binding of CGRP to the CGRP receptor follows the typical pattern established for peptide ligands to family B GPCRs in that the C-terminus of the peptide binds to the extracellular domain of CLR (in association with the extracellular domain of RAMP1), whereas the N-terminus interacts with the transmembrane (TM) domain of CLR and its associated extracellular loops. It has been suggested that all the peptide ligands for family B GPCRs share a common motif at their N-termini: a helix cap or an activation loop (Neumann *et al*., 2006; Muff *et al*., 2004; Katafuchi *et al*., 2004). The details of how the N-terminus of CGRP, using amino acid substitutions of residues 1–9 between CGRP and amylin from all species.

Although there is little experimental evidence for the role of individual amino acids at the N-terminus of CGRP, comparison of naturally occurring peptide sequences is informative. Position 1 of CGRP is not fully conserved and can either be serine or alanine (Supporting Information Fig. S1). Position 3 is usually asparagine, although in human α and mar-moset CGRP, it is aspartate. Thereafter, there is a very high degree of sequence identity up to position 14 in species from mammals to bony fish, emphasizing the conservation at the N-terminus of CGRP. Comparing CGRP with other members of the CT family of peptides including examples of the primitive cartilaginous and jawless fish (Supporting Information Fig. S2; see also Wong and Takei, 2009; Takei *et al*., 2010), the most striking feature is the conservation of position 6 in peptides apart from equine CGRP-1 [actually the equine equivalent of CRSP (Ogoshi *et al*., 2006)], CRSP 2 and 3 and AM4 in Takifugu rubripes and Tetraodon nigroviridis. AM4 has only been described in bony fish and in other species the threonine is retained. The equivalent of positions 1 and 5 is group conserved as small hydrophobic amino acids (glycine, alanine and serine). There is extensive conservation of residues 1–9 between CGRP and amylin from all species.

In this study, we investigated the structure–activity relationship of the N-terminus of CGRP, using amino acid substitutions of residues 1, 3–6 and 8–9, on CGRP receptor binding, production of cAMP and β-arrestin 2 translocation. These positions were substituted with either alanine or cysteine. Cysteine and alanine are small hydrophobic amino acids that are often group conserved along with serine in proteins. The results indicate that Ala-5 and Thr-6 are important determinants of CGRP activity at the CGRP receptor.

**Methods**

**Peptides**

All peptides are derivatives of human α CGRP, containing a Cys-2-Cys-7 disulphide bond and C-terminal amide. CGRP was purchased from American Peptide (Sunnyvale, CA, USA) or Bachem (St Helens, Merseyside, UK). [Cys]$^1$-, [Cys]$^3$-, [Cys]$^5$- and [Cys]$^9$-CGRP were synthesized by Alta Biosciences, Birmingham, UK. [Cys]$^1$-, [Ala]$^4$-, [Cys]$^5$-, [Ala]$^6$-, [Ser]$^7$-, [Asp]$^8$-, [Lys]$^9$-, [Ala]$^{10}$- and [Ala]$^{12}$-CGRP peptides were synthesized by solid phase peptide synthesis at the University of Auckland using the Fmoc/Bu method on a 0.1 mmol scale. Briefly, Rink amide amioethyl resin was prepared as described (Harris *et al*., 2011), and the peptide elongated using a CEM microwave peptide synthesizer (CEM Corporation, Matthews, NC, USA) as previously described (Harris *et al*., 2008). For [Cys]$^3$- and [Cys]$^5$-CGRP peptides, the non-disulphide cysteines were protected as the trifluorocetic acid-stable tert-butyl ethers.
For the other cysteine-substituted peptides, the non-disulphide cysteines were protected as acetylamidomethyl derivatives. The peptides were cleaved from the resin with concomitant removal of side chain protecting groups with 94.0% trifluoroacetic acid, 1.0% trisopropylsilane, 2.5% water and 2.5% ethanedithiol (v/v/v/v) for 2–3 h, precipitated with cold diethyl ether, isolated by centrifugation, dissolved in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid and lyophilized. The crude peptides were dissolved in 0.1 M Tris (pH = 8.3) at a concentration of 1 mg·mL$^{-1}$ and the oxidation (disulphide formation) allowed to proceed at room temperature open to air. Monitoring by reverse phase HPLC indicated that the reaction was typically complete after 12 h. The crude product was lyophilized, redissolved in 50.0% aqueous acetonitrile containing 0.1% trifluoroacetic acid and purified by semi-preparative reverse phase HPLC using a C18 Gemini (Phenomenex, Torrance, CA, USA) column (10 × 250 mm) at a flow rate of 5 mL·min$^{-1}$ and eluted using an appropriate gradient based on the analytical HPLC profile. Fractions containing the pure peptide were identified by electrospray mass spectrometry and/or HPLC, pooled and lyophilized. All peptides were >95% purity as judged by integration of the HPLC chromatogram at 210 nm, and peptide masses were confirmed by electrospray mass spectrometry. For [Cys$^3$]- and [Cys$^5$]-CGRP peptides, following the oxidation, the crude peptide was recovered by solid phase extraction and lyophilized. To remove the tert-butyl on the cysteine at [Cys$^3$] or [Cys$^5$], the peptide was then dissolved in trifluoroacetic acid : anisole (9:1, v/v) at a concentration of 15 mg·mL$^{-1}$, cooled to 0°C and trifluoroacetic acid : trifluromethanesulphonic acid (4:1, v/v) (0.4 mL) was added. The solution was stood at 0°C for 3 min, poured into cold ether and recovered by centrifugation. Purification as described earlier afforded the pure peptides in >95% purity.

Cell culture and transfection

SK-N-MC cells were grown in DMEM/F12 medium supplemented with 10% fetal calf serum as described previously (Poyner et al., 1998). Cos 7 cells were grown in DMEM/10% FBS and were transfected with human HA-tagged CT (a) receptors and human myc-tagged RAMP1 using polyethyleneimine and identical methods to those described previously for the CGRP receptor (Bailey and Hay, 2006). In this case, the transfection gives an AMY1(a) receptor. This protocol has been successfully used to express these receptors (Qi et al., 2008). This protocol was also used to express the CGRP receptor in Cos 7 where appropriate.

Measurement of cAMP

Production of cAMP in SK-N-MC cells for all analogues except [Cys$^3$]-hCGRP was by a radioreceptor assay, as described previously (Poyner et al., 1998). Cells were treated with increasing concentrations of the CGRP analogues for approximately 10 min. Where antagonist activity was being assessed, the cells were pretreated with peptides for 10 min prior to the addition of the CGRP derivative (Poyner et al., 1998). For [Cys$^3$]-hCGRP, a different clone of SK-N-MC cells was used and cAMP production was measured via AlphaScreen (Perkin Elmer, Wellesley, MA, USA), as described previously (Gingell et al., 2010). This assay was also used to investigate the action of all derivatives at the AMY1(a) receptor and other experiments using Cos 7 cells. In control experiments to confirm cross-compatibility of the two SK-N-MC cell clones using AlphaScreen, [Ala$^9$]-hCGRP and [Ala$^9$]-hCGRP gave similar reductions in pEC$_{50}$ values as seen when this parameter was measured by a radioreceptor assay with the other clone of cells (values of 7.58 ± 0.43 and 7.39 ± 0.16 vs. 9.00 ± 0.33 for CGRP, n = 3–4; compare with Table 1).

β-Arrestin translocation

Translocation of β-arrestin after a 2 h stimulation with peptides was measured using a DiscoveRx ‘PathHunter eXpress β-arrestin’ kit (93-0446E; Birmingham, West Midlands, UK), with CHO-K1 cells expressing the human CGRP receptor and β-arrestin 2. The role of β-arrestin 2 in CGRP receptor desensitization has been previously demonstrated (Hilairet et al., 2001; Padilla et al., 2007).

Radioligand binding

The ability of peptides to displace $^{125}$I-CGRP binding to SK-N-MC cell membranes was measured in a microcentrifugation-based binding assay, as described previously (Poyner et al., 1998).

Data analysis

GraphPad Prism 5.00 or 6.00 (GraphPad Software Inc., San Diego, CA, USA) was used for data fitting. Concentration–response curves were fitted to a sigmoidal function with Hill slope constrained to unity to obtain pEC$_{50}$ and maximum responses ($E_{\text{max}}$) values. Displacement curves were analysed to obtain pIC$_{50}$ values. Schild plots were fitted by linear regression to obtain slope and intercepts. pEC$_{50}$ and pIC$_{50}$ values were compared with that of human αCGRP using Dunnett’s test with either a normal or repeated measures one-way ANOVA, as appropriate. To assess if $E_{\text{max}}$ values differed from wild type, 95% confidence limits were calculated using Student’s t-statistic.

Materials

$[^{125}]$I-iodohistidyl$^8$ human α CGRP ($[^{125}]$I-CGRP) and $[^{3}H]$-cAMP were purchased from Perkin Elmer (Wellesley, MA, USA). Other reagents were as described previously (Barwell et al., 2011a). The SK-N-MC cells were from the European Collection of Animal Cell Cultures (Porton Down, UK), except those used for examining [Cys$^3$]-CGRP, which were a gift from Dr Fiona Marshall (Heptares, Stevenage, UK) (used for radioligand binding) or from American Type Culture Collection (used for cAMP measurements). Cells were changed due to problems with the old batch losing expression of CGRP receptors over passages, as has been documented previously (Choksi et al., 2002).

Results

The role of the side chains of positions 1, 3, 4, 8 and 9 at CGRP receptors

As expected, CGRP was a potent agonist at stimulating cAMP production in SK-N-MC cells (Figure 1; Table 1), with a pEC$_{50}$
Table 1

pKᵢ, pEC₅₀ and E\text{max} values for CGRP analogues on SK-N-MC and CHO-K1 cells

| Binding (SK-N-MC) | cAMP (SK-N-MC) | β-Arrestin (CHO-K1) |
|------------------|----------------|---------------------|
|                  | pKᵢ            | pEC₅₀               | E\text{max (% CGRP)} | pEC₅₀ | E\text{max (% CGRP)} |
|                  | Control        | CGRP analogue      | Control              | CGRP analogue | Control          | CGRP analogue | Control | CGRP analogue | Control | CGRP analogue |
| [Cys¹]            | 10.29 ± 0.05   | 9.55 ± 0.20**      | 8.80 ± 0.09          | 7.70 ± 0.28*   | 71 ± 8*          | 9.20 ± 0.16    | 8.58 ± 0.05* | 60 ± 1**      |
| [Cys²]            | 10.29 ± 0.05   | 9.54 ± 0.07**      | 8.80 ± 0.09          | 8.27 ± 0.11    | 116 ± 4          | 9.20 ± 0.16    | 8.43 ± 0.05** | 95 ± 3        |
| [Ala³]            | 10.29 ± 0.05   | 9.40 ± 0.42        | 8.80 ± 0.09          | 7.43 ± 0.26**  | 100 ± 11         | 9.20 ± 0.16    | 8.42 ± 0.04** | 73 ± 3*       |
| [Cys⁴]            | 10.43 ± 0.20   | 8.00 ± 0.26***     | 9.43 ± 0.07          | 7.18 ± 0.18†   | 23 ± 4†          | 9.20 ± 0.16    | 8.25 ± 0.04*** | 55 ± 2**      |
| [Ala⁵]            | 9.99 ± 0.17    | 8.57 ± 0.49*       | 8.48 ± 0.10          | 6.59 ± 0.20**  | 72 ± 12          | 9.20 ± 0.16    | 7.74 ± 0.28*  | 43 ± 2.0***   |
| [Cys⁶]            | 10.29 ± 0.05   | 9.33 ± 0.13**      | –                    | Undetectable   | Undetectable    | –              | Undetectable | Undetectable |
| [Ser⁷]            | 9.99 ± 0.17    | 9.12 ± 0.54*       | 8.48 ± 0.10          | 7.27 ± 0.11*   | 100 ± 10         | 9.20 ± 0.16    | 8.07 ± 0.23*  | 57.3 ± 4.8**  |
| [Asp⁸]            | 9.99 ± 0.17    | 7.50 ± 0.22**      | –                    | Undetectable   | Undetectable    | –              | Undetectable | Undetectable |
| [Lys⁹]            | 9.99 ± 0.17    | 7.76 ± 0.64**      | –                    | Undetectable   | Undetectable    | –              | Undetectable | Undetectable |
| [Ala¹⁰]           | 9.99 ± 0.17    | 9.42 ± 0.42        | 8.48 ± 0.10          | 7.73 ± 0.25**  | 104 ± 8          | 9.20 ± 0.16    | 8.81 ± 0.04   | 81 ± 3*       |
| [Ala¹ⁱ]           | 9.99 ± 0.17    | 9.28 ± 0.20        | 8.48 ± 0.10          | 7.30 ± 0.08**  | 103 ± 13         | 9.20 ± 0.16    | 8.23 ± 0.08** | 64 ± 3**      |

Values are means ± SEM, n = 3–6. ***,***P < 0.05, 0.01, 0.001 compared with CGRP, Dunnett’s test following one-way ANOVA.

*No response seen in five experiments.
within the considerable range reported in the literature (Poyner et al., 1998; Howitt et al., 2003); it also displaced ¹²⁵I-CGRP with a Ki that was 30-fold less than its pEC₅₀ (Figure 3; Table 1). It was a very potent agonist at stimulating β-arrestin 2 translocation in CHO-K1 cells (Figure 4; Table 1).

As can be seen in Table 1 and Figures 2 and 3, the introduction of a cysteine to replace aspartate in native CGRP at position 3 made little difference to potency on cAMP although there was a small (approximately sixfold) decrease in affinity. Substitution of alanine by cysteine at position 1 caused around a 10-fold decrease in potency on cAMP and modest reduction in Eₘₐₓ; these changes were accompanied by a sixfold decrease in affinity. Substitution of Thr-4, Val-8 and Thr-9 in native CGRP by alanine caused 6- to 10-fold decreases in potency on cAMP; there were no significant changes in affinity.

The analogues were also examined for their activity at stimulating β-arrestin 2 translocation in CHO-K1 cells (Figures 4 and 5; Table 1). They all showed a reduced potency of 3- to 10-fold, which was significant for every analogue except [Ala³]-CGRP. Apart from [Cys¹], they also all showed decreased Eₘₐₓ; for [Ala³]-CGRP the reduction in maximum was 20%, but for the other analogues it was approximately 40%.

Figure 1
Effects of CGRP alanine and Thr-6 analogues on cAMP production in SK-N-MC cells. Values are means ± SEM, n = 3–6 and are normalized to the response seen with CGRP.

Figure 2
Effects of CGRP cysteine analogues on cAMP production in SK-N-MC cells. Values are means ± SEM, n = 3–6 and are normalized to the response seen with CGRP.
The role of the side chains of positions 5 and 6

Substitution of Ala-5 in native CGRP by cysteine caused over a 270-fold decrease in affinity. There was a ninefold decrease in potency for stimulation of β-arrestin 2 translocation, and the $E_{\text{max}}$ was reduced by 45% compared with CGRP. The analogue was a partial agonist at stimulating cAMP production in SK-N-MC cells. A measureable response was only found in cells where the pEC$_{50}$ for CGRP was over 9, indicating tight coupling to $G_{i}$ activation. In these cells, the $E_{\text{max}}$ was reduced by almost 70%, and the potency was decreased over 100-fold (Table 1; Figures 2, 3 and 5). Consistent with its low efficacy, it could antagonize the action of CGRP. The slope of the resulting Schild plot was not significantly different from unity (1.33 ± 0.30), resulting in a pK$_{b}$ estimate for [Cys$_{5}$]-CGRP of 7.74 ± 0.12 (Figure 6C). This is in good agreement with the pK$_{b}$ of 8.00 ± 0.26 (Table 1). By comparison, in the same series of experiments, CGRP$_{8-37}$, the best characterized CGRP antagonist, had a pK$_{b}$ of 9.42 ± 0.12 ($n = 4$, data not shown).

Although this is rather high, considerable variations in affinity for this antagonist are sometimes seen (Hay et al., 2008).

Substitution of Thr-6 in native CGRP by either alanine or serine caused substantial decreases in potency in cAMP production; the potency for [Ser$_{6}$]-CGRP was reduced over 10-fold and for [Ala$_{6}$]-CGRP there was almost a 100-fold reduction in potency with a trend towards a reduced $E_{\text{max}}$. There were reductions in affinity that was the largest for [Ala$_{6}$]-CGRP (30-fold). There were also decreases in potency when β-arrestin 2 translocation was examined and both the

Figure 3
Inhibition of [¹²⁵I]-CGRP binding by CGRP analogues in SK-N-MC cell membranes. Values are means ± SEM, n = 3–6.

Figure 4
Stimulation of β-arrestin 2 translocation by CGRP alanine and Thr-6 analogues in CHO-K1 cells. Values are means ± SEM, n = 3 and are normalized to the response seen with CGRP.
analogue were partial agonists, with the $E_{\text{max}}$ to [Ser6]-CGRP reduced by 40% and that to [Ala6]-CGRP by almost 60% (Figure 4; Table 1).

Substitution of Thr-6 by cysteine, aspartate and lysine had more profound effects. [Asp6]- and [Lys6]-CGRP both had over 100-fold decreases in affinity. They also failed to stimulate cAMP production or $\beta$-arrestin 2 translocation at concentrations of up to either 1 $\mu$M ($\beta$-arrestin 2) or 10 $\mu$M (cAMP). The loss of efficacy was confirmed by examining the cAMP response to CGRP in the presence of 10 $\mu$M of either of these agents. Both produced small but significant rightward shifts in the concentration–response curves to CGRP with no suppression of the $E_{\text{max}}$ (Figure 6A): pEC50 for CGRP, 8.66 $\pm$ 0.19; with 10 $\mu$M [Asp6]-CGRP, 8.04 $\pm$ 0.04 ($P < 0.05$) and with 10 $\mu$M [Lys6]-CGRP, 8.11 $\pm$ 0.09 ($P < 0.01$), indicating that they were acting as competitive antagonists. For [Cys6]-CGRP, the loss in affinity was only fivefold (Figure 3; Table 1), but as with the aspartate and lysine derivatives, it was also unable to stimulate either production of cAMP or translocation of $\beta$-arrestin 2. To confirm the loss of efficacy at cAMP production, the analogue was used as an antagonist to shift the concentration–response curve to CGRP (Figure 6B); the Schild plot had a straight line with a slope not significantly different from unity (0.98 $\pm$ 0.20) and a pA2 of 8.08 $\pm$ 0.13 ($n=3$). This indicates that it behaves as a competitive antagonist.

**Actions of analogues at stimulating cAMP production at the AMY1(a) receptor**

The structure–activity relationship with the analogues at the CGRP receptor was also broadly apparent when the deriva-

| CGRP analogue | pEC50  | $E_{\text{max}}$ (% CGRP) |
|---------------|--------|---------------------------|
| Control       | 9.22 $\pm$ 0.13 | 8.90 $\pm$ 0.03          |
| [Cys5]        | 9.66 $\pm$ 0.15 | 7.78 $\pm$ 0.12***        |
| [Ala6]        | 9.76 $\pm$ 0.15 | 7.00 $\pm$ 0.44**         |
| [Cys6] –     | Undetectable |                           |
| [Ser6]        | 9.76 $\pm$ 0.15 | 9.25 $\pm$ 0.03           |
| [Asp6] –     | Undetectable |                           |
| [Lys6] –     | Undetectable |                           |
| [Ala8]        | 9.22 $\pm$ 0.13 | 8.97 $\pm$ 0.07           |
| [Ala9]        | 9.22 $\pm$ 0.13 | 8.21 $\pm$ 0.32**         |

Values are means $\pm$ SEM, $n=3$–6. *,**,***, $P < 0.05, 0.01, 0.001$ compared with CGRP, Dunnett’s test followed by one-way ANOVA or Student’s $t$-test, as appropriate.

tives were used to stimulate cAMP production in Cos 7 cells via the AMY1(a) receptor (Table 2; Figures 7 and 8), which uses the CT receptor rather than CLR. [Cys5]-CGRP was 75-fold less potent than CGRP and was a partial agonist. In Cos 7 cells transfected with CLR and RAMP1 (Figure 7), [Cys5]-CGRP appeared as a full agonist ($E_{\text{max}}$ 90 $\pm$ 8% of that of CGRP) albeit
much less potent than CGRP itself (pEC50 for CGRP, 9.66 ± 0.22; pEC50 for [Cys5]-CGRP, 7.90 ± 0.20). [Ala9]-hαCGRP was 10-fold less potent than CGRP. [Ala6]-CGRP was over 700-fold less potent than CGRP and it was also a partial agonist. There were no consistent stimulations of cAMP production seen with [Cys6]-, [Asp6]- or [Lys6]-CGRP. The potency of [Ser6]-CGRP was not significantly different from that of CGRP, and although there was a trend for a reduced Emax, this did not reach significance and [Ala8]-CGRP also behaved like CGRP.

Discussion

This paper assesses the contribution of the first nine residues of CGRP, excluding the cysteines at positions 2 and 7 to receptor binding and activation. The results highlight the contribution of residues in the middle and C-terminal portions of this part of CGRP, especially Ala-5 and Thr-6.

Substitution of the alanine at position 5 by cysteine causes large decreases in affinity and efficacy at cAMP production in SK-N-MC cells (Figure 8). The affinity loss is large, especially considering that there is only around a 10-fold loss typically found for deletion of the first seven amino acids to give CGRP8–37 (Chiba et al., 1989; Watkins et al., 2013). It seems that the sulfhydryl group at position 5 results in the N-terminus adopting a conformation that impairs the binding of the rest of the CGRP molecule. In CHO-K1 cells, the reduction in efficacy seen with [Cys5]-CGRP at stimulating β-arrestin translocation is smaller than that seen on cAMP in SK-N-MC cells, although this may simply reflect the better receptor coupling seen in the former cells, as discussed below.

Substitution of Thr-6 by cysteine gave only a modest reduction in affinity, but all measurable efficacy was lost for coupling to both Gs and translocation of β-arrestin 2. Threonine is able to take part in both hydrogen bonds and hydrophobic interactions, and the structure–activity relationship revealed in this study suggests that both are likely to be important. The failure of serine to substitute for threonine at CGRP is striking as the position of the hydroxyl group in the two analogues is identical; they differ only in that threonine has an extra methyl group on the β-carbon. However, these data are consistent with the fact that serine is not found in position 6 in any known native member of the CT/CGRP family (Supporting Information Figs S1 and S2). It would appear likely that the threonine fits into a tightly constrained pocket, where both the methyl and the hydroxyl groups are important. The probability is that the hydroxyl group is involved in a hydrogen bond; the methyl group may pack against a hydrophobic group. The poor binding and lack of efficacy seen with both the lysine and aspartate derivatives may imply that the partner for threonine is uncharged; however, it is also possible that steric hindrance could explain the large effects with these derivatives.

Throughout the CT/CGRP family, positions 5 and 6 are highly conserved, suggesting that they may have similarly important roles across all members of the family. The equivalent of position 5 is group conserved as a small hydrophobic (or weakly hydrophilic) residue: alanine, serine or glycine, except for AM4 sequences where larger hydrophobic residues are present. The equivalent of position 6 is always a threonine except in AM4 and CRSP 2 and 3, and there is some doubt whether these three peptides are agonists at CLR-based receptors. Porcine CRSP 2 and 3 do not stimulate cAMP production at CLR or CT receptor expressed in Cos 7 cells, with or without RAMPs (Katafuchi et al., 2009), and dog CRSP 2 has no action on the CT receptor of LLC-PK cells (Ogoshi et al., 2006). There is no information on the biological activity of AM4, although as the mRNA is present in large amounts in the skin of Takifugu, it has been suggested that it may have an antimicrobial role (Ogoshi et al., 2003).

There is no previous work on position 5 of CGRP, but one study has looked at position 6. [Val4]-CGRP was inactive at stimulating cAMP production in porcine iris ciliary body (Heino et al., 1998), in agreement with the conclusions from this study that the residue cannot be substituted without loss of activity. Interestingly, an AM derivative, where replace-

Figure 6

Antagonism of cAMP production by CGRP with (A) [Asp6] and [Lys6]- and (B) [Cys5]-CGRP, (C) [Cys5]-CGRP in SK-N-MC cells.
ment of the equivalent of Thr-6 (Thr-20) was reported to reduce potency on blood pressure (Kuwasko et al., 2011), is consistent with this being an important residue for all members of the CT/CGRP family. Similarly, substitution of Thr-6 in amylin by alanine resulted in reduced activity of the peptide (Roth et al., 2008).

Substitution of the remaining residues of CGRP had generally rather modest but significant effects on binding and potency at stimulating cAMP and β-arrestin 2 translocation. There was a good correlation between the potency of the analogues at stimulating cAMP and their binding affinities ($r^2 = 0.78$), although the correlation between binding and β-arrestin translocation was much weaker ($r^2 = 0.40$, Figure 8). Asp-3 was the least significant substitution with cysteine only causing a small reduction in affinity. A photoaffinity probe can be accommodated here with retention of high affinity potency at stimula...
binding (Stangl et al., 1993), consistent with the side chain of the residue sitting in an exposed area when CGRP is bound to its receptor, although it is highly conserved in CGRP as either aspartate or asparagine. Substitutions at all the remaining positions impaired the potency of the analogue at stimulating cAMP and efficacy at β-arrestin 2 translocation, indicating that their side chains have roles in receptor activation. There was little evidence for selective activation of either of the two pathways with any of these analogues (Figure 8), although as the responses were compared in different cell lines, quantitative comparison of efficacy measures is difficult.

As CGRP8–37 is an antagonist, it is surprising that substitutions at positions 8 and 9 apparently reduced efficacy. However, a photoaffinity probe attached to position 8 of salmon CT can label a residue at the top of the third extracellular loop (Dong et al., 2004). Potentially, residues in this part of the ligand could interact directly with the juxtamembrane part of the receptor and enhance the ability of the disulphide-bonded ring to cause receptor activation. Thr-9 may, alternatively, act indirectly by helping to stabilize a conformation of the N-terminus of CGRP that is favourable for binding by making a hydrogen bond to connect with an appropriate H-bond acceptor located between residues 1 and 7 on the disulphide-bonded ring. There is support for this from NMR evidence (Breeze et al., 1991) and molecular dynamics simulations (D. L. Rathbone and D. R. Poyner, unpublished) as well as the position of this residue within the N-cap proposed by Watkins et al. (2012). Whatever the mechanism is, it is clear that agonist activity is influenced by residues outside the disulphide-bonded loop. Interestingly, CGRP1–7 is reported to be an antagonist (Dennis et al., 1989) whereas CGRP1–12 can mimic the hypotensive effects of CGRP and so may be an agonist (Maggi et al., 1990); this raises the possibility that the 1–7 ring requires other residues such as Val-8 and Thr-9 in order to activate the receptor.

The CGRP analogues showed a similar pattern of activity for stimulation of cAMP production on the AMY1(a) receptor as they did on the CGRP receptor (Figure 8D; $r^2 = 0.70$); CGRP is a potent agonist at both receptors (Hay et al., 2005). In particular, Ala-5 and Thr-6 were both important for agonist action. Potency was also influenced by substitution of Thr-9; this is conserved between CGRP and amylin. Thus, the same general determinants of receptor activity are likely to operate at both receptors for coupling to Gs. A detailed comparison of the importance of individual residues at stimulating the AMY1(a) and the CGRP receptor is complicated by the fact that the AMY1(a) receptor was expressed in Cos 7 cells and the CGRP receptor was expressed in SK-N-MC cells. There seems to be better coupling of the CGRP receptor to Gs in the Cos 7 cells compared with the SK-N-MC cells. There seems to be better coupling of the CGRP receptor to Gs in the Cos 7 cells compared with the SK-N-MC cells. There seems to be better coupling of the CGRP receptor to Gs in the Cos 7 cells compared with the SK-N-MC cells. There seems to be better coupling of the CGRP receptor to Gs in the Cos 7 cells compared with the SK-N-MC cells. There seems to be better coupling of the CGRP receptor to Gs in the Cos 7 cells compared with the SK-N-MC cells. There seems to be better coupling of the CGRP receptor to Gs in the Cos 7 cells compared with the SK-N-MC cells. There seems to be better coupling of the CGRP receptor to Gs in the Cos 7 cells compared with the SK-N-MC cells. There seems to be better coupling of the CGRP receptor to Gs in the Cos 7 cells compared with the SK-N-MC cells. There seems to be better coupling of the CGRP receptor to Gs in the Cos 7 cells compared with the SK-N-MC cells. There seems to be better coupling of the CGRP receptor to Gs in the Cos 7 cells compared with the SK-N-MC cells. There seems to be better coupling of the CGRP receptor to Gs in the Cos 7 cells compared with the SK-N-MC cells.

Although it is not possible on the basis of the data in this study to propose a model of how CGRP binds to its receptor, a number of general points can be made. The first and third extracellular loops of CLR play only relatively minor roles in binding CGRP (Barwell et al., 2011a); the second extracellular loop is much more significant (Barwell et al., 2012; Woolley et al., 2013). We have previously modelled CGRP bound to the TM domain of CLR, in close proximity to CLR (Woolley et al., 2013). This model is consistent with the data in the current study. The side chain of Ala-5 and the methyl group of Thr-6 are buried in the interface between TM helices 5 and

Figure 8
Correlations between effects of CGRP analogues. (A) Binding v cAMP production, CGRP receptor; (B) beta-arrestin translocation v cAMP production, CGRP receptor; (C) binding v beta-arrestin translocation, CGRP receptor; (D) cAMP production, AMY1(a) receptor v CGRP receptor. Values are the Δlog pEC50 or Δlog pKi, where the value for the analogue was subtracted from that of its paired control. Thus, 1 represents a 10-fold reduction, 2 represents a 100-fold reduction and 3 for 1000-fold. Where activity could not be detected no value is included.

424 British Journal of Pharmacology (2014) 171 415–426
6; the hydroxyl group on Thr-6 could H-bond to a backbone carbonyl on the C-terminal portion of the second extracellular loop. Asp-3 is in an unhindered position, free from steric constraints. Ala-1 points between the top of the first extracellular loop and TM helix 1, allowing N-terminal extensions of the peptide. The residues that interact with CGRP in the CLR as part of the CGRP receptor are likely to be conserved in the CT receptor.

In conclusion, this paper provides new information on the structural requirements needed for agonist activity of CGRP. Alanine at position 5 and threonine at position 6 are particularly important for receptor activation. CGRP positions 1, 4, 8 and 9 also influence agonist activity. It would be interesting to evaluate analogues based on the pentapeptide CGRP₅₋₉, as this may contain the key components needed for receptor activation.

**Acknowledgements**

D. R. P. was supported by grants from the Welcome Trust 088212 and 091496 and also from the Aston Research Centre for Healthy Aging (ARCHA). We thank the Maurice Wilkins Centre for Molecular Biodiscovery for support for peptide synthesis. D. L. H. and M. A. B. were supported by the Marsden Fund Council from government funding, administered by the Royal Society of New Zealand.

**Conflict of interest**

None.

**References**

Alexander SPH et al. (2013). The Concise Guide to PHARMACOLOGY 2013/14: Overview. Br J Pharmacol 170: 1449–1867.

Bailey RJ, Hay DL (2006). Pharmacology of the human CGRP₁ receptor in Cos 7 cells. Peptides 27: 1367–1375.

Bailey RJ, Walker CS, Ferner AH, Loomes KM, Prijic G, Halim A et al. (2012). Pharmacological characterisation of rat amylin receptors: implications for the identification of amylin receptor subtypes. Br J Pharmacol 166: 151–167.

Barwell J, Conner A, Poyner DR (2011a). Extracellular loops 1 and 3 and their associated transmembrane regions of calcitonin receptor-like receptor are needed for CGRP receptor function. Biochim Biophys Acta 1813: 1906–1916.

Barwell J, Gingell JJ, Watkins HA, Archbold JK, Poyner DR, Hay DL (2011b). Calcitonin and calcitonin receptor-like receptors: common themes with family B GPCRs? Br J Pharmacol 166: 51–65.

Barwell J, Woolley MJ, Wheatley M, Conner AC, Poyner DR (2012). The role of the extracellular loops of the CGRP receptor, a family B GPCR. Biochem Soc Trans 40: 433–437.

Breeze AL, Harvey TS, Bazzo R, Campbell ID (1991). Solution structure of human calcitonin gene-related peptide by 1H NMR and distance geometry with restrained molecular dynamics. Biochemistry 30: 575–582.

Chiba T, Yamaguchi A, Yamatani T, Nakamura A, Morishita T, Inui T et al. (1989). Calcitonin gene-related peptide receptor antagonist human CGRP-(8-37). Am J Physiol 256 (2 Pt 1): E331–E335.

Choksi T, Hay DL, Legon S, Poyner DR, Hagner S, Bloom SR et al. (2002). Comparison of the expression of calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs) with CGRP and adrenomedullin binding in cell lines. Br J Pharmacol 136: 784–792.

Christopoulos G, Perry KJ, Morfis M, Tilakaratne N, Gao Y, Fraser NJ et al. (1999). Multiple amylin receptors arise from receptor activity-modifying protein interaction with the calcitonin receptor gene product. Mol Pharmacol 56: 235–242.

Conner AC, Hay DL, Howitt SG, Kilk K, Langel U, Wheatley M et al. (2002). Interaction of calcitonin-gene-related peptide with its receptors. Biochem Soc Trans 30: 451–455.

Dennis T, Fournier A, St Pierre S, Quirion R (1989). Structure–activity profile of calcitonin gene-related peptide in peripheral and brain tissues. Evidence for receptor multiplicity. J Pharmacol Exp Ther 251: 718–725.

Dong M, Pinon DI, Cox RF, Miller LJ (2004). Molecular approximation between a residue in the amino-terminal region of calcitonin and the third extracellular loop of the class B G protein-coupled calcitonin receptor. J Biol Chem 279: 31177–31182.

Gingell JJ, Qi T, Bailey RJ, Hay DL (2010). A key role for tryptophan 84 in receptor activity-modifying protein 1 in the amylin 1 receptor. Peptides 31: 1400–1404.

Hakala JM, Valo T, Vihavainen S, Hermonen J, Heino P, Halme M et al. (1994). Constrained analogues of the calcitonin gene-related peptide. Biochem Biophys Res Commun 202: 497–503.

Harris PWR, Williams GM, Shepherd P, Brimble MA (2008). The synthesis of phosphopeptides using microwave-assisted solid phase peptide synthesis. Int J Pept Res Ther 14: 387–392.

Harris PWR, Yang SH, Brimble MA (2011). An improved procedure for the preparation of aminomethyl polystyrene resin and its use in solid phase (peptide) synthesis. Tetrahedron Lett 52: 6024–6026.

Hay DL, Christopoulos G, Christopoulos A, Poyner DR, Sexton PM (2005). Pharmacological discrimination of calcitonin receptor: receptor activity-modifying protein complexes. Mol Pharmacol 67: 1655–1665.

Hay DL, Poyner DR, Quirion R (2008). International Union of Pharmacology. LXIX. Status of the calcitonin gene-related peptide subtype 2 receptor. Pharmacol Rev 60: 143–145.

Heino P, Oksala O, Palkama A, Valo T, Vihavainen S, Koskinen A et al. (1998). Binding of CGRP analogs and their effect on adenylate cyclase activity in porcine iris-ciliary body. J Ocul Pharmacol Ther 14: 543–554.

Hilaiaret S, Belanger C, Bertrand J, Laperriere A, Foord SM, Bouvier M (2001). Agonist-promoted internalization of a ternary complex between calcitonin receptor-like receptor, receptor activity-modifying protein 1 (RAMP1), and beta-arrestin. J Biol Chem 276: 42182–42190.

Howitt SG, Kilk K, Wang Y, Smith DM, Langel U, Poyner DR (2003). The role of the 8-18 helix of CGRP8-37 in mediating high affinity binding to CGRP receptors; coulombic and steric interactions. Br J Pharmacol 138: 325–332.

Katafuchi T, Yasue H, Osaki T, Minamino N (2009). Calcitonin and calcitonin receptor-like receptors: common interactions. Br J Pharmacol 138: 325–332.
Kuwasako K, Kitamura K, Nagata S, Hikosaka T, Takei Y, Kato J (2011). Shared and separate functions of the RAMP-based adrenomedullin receptors. Peptides 32: 1540–1550.

Lang M, De Pol S, Baldauf C, Hofmann HJ, Reiser O, Beck-Sickinger AG (2006). Identification of the key residue of calcitonin gene-related peptide (CGRP) 27–37 to obtain antagonists with picomolar affinity at the CGRP receptor. J Med Chem 49: 616–624.

Maggi CA, Rovero P, Giuliani S, Evangelista S, Regoli D, Meli A (1990). Biological activity of N-terminal fragments of calcitonin gene-related peptide. Eur J Pharmacol 179: 217–219.

McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N et al. (2007). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 393: 333–339.

Muff R, Buhlmann N, Fischer JA, Born W (1999). An amylin receptor is revealed following co-transfection of a calcitonin receptor with receptor activity modifying proteins-1 or -3. Endocrinology 140: 2924–2927.

Neumann JM, Couvineau A, Murail S, Lacapere JJ, Jamin N, Laburthe M (2008). Class-B GPCR activation: is ligand helix-capping the key? Trends Biochem Sci 33: 314–319.

Ogoshi M, Inoue K, Takei Y (2003). Identification of a novel adrenomedullin gene family in teleost fish. Biochem Biophys Res Commun 311: 1072–1077.

Ogoshi M, Inoue K, Naruse K, Takei Y (2006). Evolutionary history of the calcitonin gene-related peptide family in vertebrates revealed by comparative genomic analyses. Peptides 27: 3154–3164.

Padilla BE, Cottrell GS, Roosterman D, Pikios S, Muller L, Steinhoff M et al. (2007). Endothelin-converting enzyme-1 regulates endosomal sorting of calcitonin receptor-like receptor and beta-arrestins. J Cell Biol 179: 981–997.

Poyner DR, Andrew DP, Brown D, Bose C, Hanley MR (1992). Pharmacological characterization of a receptor for calcitonin gene-related peptide on rat, L6 myocytes. Br J Pharmacol 105: 441–447.

Poyner DR, Soomets U, Howitt SG, Langel U (1998). Structural determinants for binding to CGRP receptors expressed by human SK-N-MC and Col 29 cells: studies with chimeric and other peptides. Br J Pharmacol 124: 1659–1666.

Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W et al. (2002). International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol Rev 54: 233–246.

Qi T, Christopoulos G, Bailey RJ, Christopoulos A, Sexton PM, Hay DL (2008). Identification of N-terminal receptor activity-modifying protein residues important for calcitonin gene-related peptide, adrenomedullin, and amylin receptor function. Mol Pharmacol 74: 1059–1071.

Recober A, Russo AF (2009). Calcitonin gene-related peptide: an update on the biology. Curr Opin Neurol 22: 241–246.

Roth JD, Mack CM, Soares CJ, Ghosh SS, Parkes DJ (2008). Amylin-based pharmacotherapy – past, present & future. Immun Endoc Metab Agents Med Chem 8: 317–324.

Saha S, Waugh DJ, Zhao P, Abel PW, Smith DD (1998). Role of conformational constraints of position 7 of the disulphide bridge of h-alpha-CGRP derivatives in their agonist versus antagonist properties. J Pept Res S2: 112–120.

Stangl D, Muff R, Schmolck C, Fischer JA (1993). Photoaffinity labeling of rat calcitonin gene-related peptide receptors and adenylyl cyclase activation: identification of receptor subtypes. Endocrinology 132: 744–750.

Takei Y, Hyodo S, Katafuchi T, Minamino N (2004). Novel fish-derived adrenomedullin in mammals: structure and possible function. Peptides 25: 1643–1656.

Takei Y, Ogoshi M, Wong MKS, Nobata S (2010). Molecular and functional evolution of the adrenomedullin family in vertebrates: what do fish studies tell us. In: Hay DL, Dickerson IM (eds). The Calcitonin Gene-Related Peptide Family: Form, Function and Future Perspectives. Springer: Heidelberg, pp. 1–21.

Thiebaud D, Akatsu T, Yamashita T, Suda T, Noda T, Martin RE et al. (1991). Structure–activity relationships in calcitonin gene-related peptide: cyclic AMP response in a preosteoblast cell line (KS-4). J Bone Miner Res 6: 1137–1142.

Watts HA, Au M, Hay DL (2012). The structure of secretin family GPCR peptide ligands: implications for receptor pharmacology and drug development. Drug Discov Today 17: 1006–1014.

Watts HA, Rathbone DL, Barwell J, Hay DL, Poyner DR (2013). Structure–activity relationships for alpha calcitonin gene-related peptide. Br J Pharmacol 170: 1308–1322.

Wong MK, Takei Y (2009). Cyclostome and chondrichthyan adrenomedullins reveal ancestral features of the adrenomedullin family. Comp Biochem Physiol B Biochem Mol Biol 154: 317–325.

Wooley MJ, Watkins HA, Karakullukcu ZG, Barwell J, Smith KJ, Hay DL et al. (2013). The role of ECL2 in CGRP receptor activation: a combined modelling and experimental approach. J R Soc Interface 10: 20130589.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

http://dx.doi.org/10.1111/bph.12464

Figure S1 Alignment of CGRP sequences.

Figure S2 N-terminal regions of CGRP/calcitonin family members, aligned over CGRP1-20.

Table S1 Pharmacology of selected CLR/RAMP and CTR/RAMP complexes.