Novel Peptide Antagonists of Adrenomedullin and Calcitonin Gene-Related Peptide Receptors: Identification, Pharmacological Characterization, and Interactions with Position 74 in Receptor Activity-Modifying Protein 1/3

Samuel D. Robinson, Jacqueline F. Aitken, Richard J. Bailey, David R. Poyner, and Debbie L. Hay

School of Biological Sciences, University of Auckland, Auckland, New Zealand (S.D.R., J.F.A., R.J.B., D.L.H.); and School of Life and Health Sciences, Aston University, Birmingham, United Kingdom (D.R.P.)

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ABSTRACT

Human adrenomedullin (AM) is a 52-amino acid peptide belonging to the calcitonin peptide family, which also includes calcitonin gene-related peptide (CGRP) and AM2. The two AM receptors, AM1 and AM2, are calcitonin receptor-like receptor (CL)/receptor activity-modifying protein (RAMP) (RAMP2 and RAMP3, respectively) heterodimers. CGRP receptors comprise CL/RAMP1. The only human AM receptor antagonist (AM22–52) is a truncated form of AM; it has low affinity and is only weakly selective for AM1 over AM2 receptors. To develop novel AM receptor antagonists, we explored the importance of different regions of AM in interactions with AM1, AM2, and CGRP receptors. AM22–52 was the framework for generating further AM fragments (AM26–52 and AM30–52), novel AM/CGRP chimeras (C1–C5 and C9), and AM/AM2 chimeras (C6–C8). cAMP assays were used to screen the antagonists at all receptors to determine their affinity and selectivity. Circular dichroism spectroscopy was used to investigate the secondary structures of AM and its related peptides. The data indicate that the structures of AM, AM2, and αCGRP differ from one another. Our chimeric approach enabled the identification of two nonselective high-affinity antagonists of AM1, AM2, and CGRP receptors (C2 and C6), one high-affinity antagonist of AM2 receptors (C7), and a weak antagonist selective for the CGRP receptor (C5). By use of receptor mutagenesis, we also determined that the C-terminal nine amino acids of AM seem to be responsible for its interaction with Glu74 of RAMP3. We provide new information on the structure-activity relationship of AM, αCGRP, and AM2 and how AM interacts with CGRP and AM2 receptors.

Human adrenomedullin (AM) is a 52-amino acid peptide belonging to the calcitonin family of peptides, which also includes calcitonin gene-related peptide (CGRP) and AM2 (also known as intermedin). The calcitonin peptide family shows weak homology at the level of the primary sequence, but there are stronger relationships at the secondary structure level. Each member of the family has an N-terminal ring structure and an amidated carboxyl terminus. Both of these structures are critical for receptor binding and subsequent signaling (Eguchi et al., 1994; Conner et al., 2002). Truncation of the N-terminal ring structure produces antagonist peptides in all family members. The ring structure of AM is created by the formation of a disulfide bond between two cysteine residues at positions 16 and 21 of the peptide (Eguchi et al., 1994). Truncation of AM yields the AM antagonist AM22–52. Interfering with the C-terminal amidated tyrosine residue of AM results in a peptide with reduced receptor affinity (Eguchi et al., 1994). AM13–52 and AM15–52 are both agonists with similar affinity to full-length AM, however, little is known structurally about AM.

The combination of the calcitonin receptor-like receptor (CL) with a receptor activity-modifying protein (RAMP) constitutes receptors that bind AM (Poyner et al., 2002). CL is unusual for a G protein-coupled receptor in that it cannot function as a receptor by itself and requires heterodimerization for translocation to the cell surface (Sexton et al., 2006). Three human RAMPs have been cloned: RAMP1, RAMP2, and RAMP3. CL/RAMP complexes can bind each of the peptides AM, AM2, and CGRP but with different affinities (Poyner et al., 2002; Hay et al., 2005). CL/RAMP1 is a high-affinity CGRP receptor, but it also binds AM with lower affinity. CL/RAMP2 and CL/RAMP3 recognize AM with highest affinity and are therefore AM1 and AM2 receptors, respectively. Both receptors also recognize...
affinity, and the pharmacology looks more AM2 receptor-like. If the opposite substitution is made, replacing Trp74 with glutamic acid at position 74 (Glu74) in RAMP3 may be crucial for determining high-affinity AM binding at the AM2 receptor (Qi et al., 2008). If this residue is substituted with tryptophan (found in the equivalent position of RAMP1; Trp74), AM potency and affinity are decreased and the pharmacology looks more CGRP receptor-like in profile.

There is accumulating evidence that through the AM1 receptor expressed in endothelial cells, AM promotes angiogenesis. Genetic models of AM, CL, and RAMP2 have provided strong evidence for AM signaling being required for proper blood and lymphatic vascular development (Fritz-Six et al., 2008; Ichikawa-Shindo et al., 2008). AM is also a potent vasodilator. Therefore, AM receptors represent therapeutically useful targets (García et al., 2006).

To date, AM22–52 is the only AM receptor antagonist to be validated. AM22–52, however, has only weak affinity (pA2 = 7.34) for the AM1 receptor and even weaker affinity for the AM2 receptor (pA2 = 6.73) (Hay et al., 2003). This degree of selectivity is not sufficient to be able to distinguish these AM receptor subtypes in vivo. In fact, there are no pharmacological tools that can be used to determine the relative functions of AM1 and AM2 receptors. Furthermore, AM is little studied in terms of structure-function relationships. Therefore, the aim of this study was to explore the importance of different regions of the AM peptide in interactions with AM1 and AM2 receptors for antagonist affinity. We used the antagonist AM22–52 as the framework for generating a series of novel antagonists, including AM fragments, AM/aCGRP chimeras (C1–C5 and C9), and AM/AM2 chimeras (C6–C8) (Fig. 1). The selection of the specific regions of AM2 and aCGRP to be incorporated into the chimeras was based on where their alignment with AM shows greatest sequence divergence. Incorporating the regions of highest divergence was most likely to increase the chances of altering the affinity and/or selectivity of the peptides. To our knowledge, there are no data reporting the secondary structure of AM or AM2, so we have also investigated this using circular dichroism spectroscopy, along with comparisons to our novel peptides and aCGRP, which has a known structure.

### Materials and Methods

**Cell Culture, Transfection, and Expression Constructs.** Culture of COS-7 cells was performed as described previously (Bailey and Hay, 2006). SK-N-MC cells were cultured in the same manner as CGRP with lower affinity, with the AM2 receptor having a higher affinity compared with the AM1 receptor, depending on the species (Poyner et al., 2002; Hay et al., 2003). It has been reported that AM2 was a nonselective agonist at the three CL/RAMP complexes (Roh et al., 2004), but recent evidence suggests that AM2 can show higher affinity for CL/RAMP3 compared with CL/RAMP1 (Qi et al., 2008). The mechanism of AM binding to its receptors is still poorly defined but is most likely to involve AM binding in a pocket formed from the extracellular N termini of CL and RAMP2 or RAMP3 (for review, see Hay et al., 2006). Accumulating data suggest that glutamic acid at position 74 (Glu74) in RAMP3 may be crucial for determining high-affinity AM binding at the AM2 receptor (Qi et al., 2008). If this residue is substituted, with tryptophan (found in the equivalent position of RAMP1; Trp74), AM potency and affinity are decreased and the pharmacology looks more CGRP receptor-like.

**COS-7 cells.** Cells were grown as monolayers in a humidified incubator at 37°C under 5% CO2. The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing −7.5% heat-inactivated fetal bovine serum with penicillin/streptomycin antibiotics in 75-cm² flasks. Cells required subculturing when they reached confluence, approximately every 2 to 3 days. SK-N-MC cells were plated so that they were ~90% confluent on the day of the assay, whereas COS-7 cells were plated out so that they were between 60 and 90% confluent on the day of transfection. Transfections were undertaken using polyethyleneimine as described previously (Bailey and Hay, 2006). Cells were transfected with human HA-CL and either human RAMP2, human RAMP3, human RAMP1, human RAMP3 incorporating the mutation E74W, or human RAMP1 incorporating the mutation W74E (all in pcDNA3.1). E74W was described previously (Qi et al., 2008). W74E RAMP1 was generated using site-directed mutagenesis. Primer sequences were as follows: forward, 5'-GGC CGA CTG CAC CGA GCA CAT GGC GGA GAA GC-3' and reverse, 5'-GCT TCT CCG CCA TGT GCT CGG TGC AGT CGG-3'. The mutation was confirmed by sequencing in the DNA sequencing facility, School of Biological Sciences, University of Auckland (Auckland, New Zealand).

**cAMP Assay.** The cAMP assay was performed as described previously (Bailey and Hay, 2006). In brief, cells were serum-deprived in 50 µl/well Dulbecco's modified Eagle's medium containing 1 mM isobutyl methylxanthine and 0.1% bovine serum albumin for 30 min and then treated with agonist (25 µM/dish) for 5 min in the absence or presence of antagonist (25 µM) and incubated at 37°C for 15 min. We used 50 µM forskolin (Tocris Bioscience, Ellville, MO) as a positive control and to normalize the receptor responses. cAMP was extracted by addition of ice-cold absolute ethanol (50 µl/well) and assayed by radioreceptor assay as described previously (Bailey and Hay, 2006).

**Circular Dichroism Spectroscopy.** Circular dichroism measurements were carried out using a π-Star 180 spectrometer (Applied Photophysics, Leatherhead, UK). AM22–52 (50 µM) was initially tested in three different solutions: dH2O, phosphate buffer (50 mM Na2HPO4, pH 7.4, in dH2O), or 50% trifluoroethanol (TFE). It showed no secondary structure organization in either dH2O or phosphate buffer (data not shown); however, in 50% TFE secondary structure was obtained (Fig. 2). Therefore, 50% TFE solution was used for the remaining experiments, with AM22–52 as the benchmark. Peptides were dissolved in 50% TFE at a concentration of 50 µM (or 25 µM for the full-length peptides). CD spectra were collected from 250 to 180 nm at 0.5-nm intervals with a bandwidth of 1 nm and data collection time of 1 s at each wavelength. Spectra were collected under nitrogen gas.
pared with AM22–52. The three peptides AM, AM2, and (CGRP8–37) caused a large change in secondary structure, and AM22–52, AM26–52, and AM2 were also (H11022 troscopy data were analyzed for secondary structure content using from unity. Unpaired nist tests were used to compare antagonist pEC50 values for each. A p44 value for each antagonist was then determined using global Schild analysis (Hay et al., 2005). Schild slopes were not significantly different for each antagonist was fitted to obtain pEC50 values for each. A p

CGRP8–37 were purchased from American Peptide Co., Inc. (Sunnyvale, CA). All peptides used were of human sequence and were phy-purified. All peptides used were of human sequence and were confirmed via matrix-assisted laser desorption ionization/time of flight mass spectrometry.

Data Analysis and Statistical Procedures. Data analysis, statistical interpretation, curve fitting, and graphing were undertaken using Prism 4.03 or 5.00 (GraphPad Software Inc., San Diego, CA). Raw data generated in the cAMP assay were normalized to the control on each plate. cAMP data from agonist alone and agonist + antagonist were fitted to obtain pEC50 values for each. A p

Results

Secondary Structure Composition. CD spectroscopy was used to compare secondary structure composition of peptides. The full-length peptides (AM, AM2, and αCGRP) were compared with each other and with their respective antagonist fragments (AM22–52 and CGRP8–37). C1 to C9 were compared with AM22–52. The three peptides AM, AM2, and αCGRP differed markedly in α-helix, β-sheet, and random coil content, which is not surprising given their lack of sequence homology. AM consists of 28% α-helix, AM2 shows 44% α-helix, and αCGRP contains 88% α-helix (Fig. 2; Table 1). Truncation of the N-terminal 21 residues of AM (AM22–52) did not change the secondary structure of the peptide; however, loss of the N-terminal seven residues of CGRP (CGRP8–37) caused a large change in secondary structure, with a major loss of α-helical content and a compensatory increase in β-sheet (Table 1). The similarities in the amino acid sequences of AM26–52, AM30–52, and C1 are reflected in

<table>
<thead>
<tr>
<th>AM</th>
<th>AM2p–52</th>
<th>αCGRP</th>
<th>CGRP8–37</th>
<th>AM2</th>
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<td>β-Sheet</td>
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<td>Random coil</td>
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</table>

Fig. 2. CD spectra of AM, αCGRP, and AM2.

AM Fragments. As expected from previous work, AM22–52 was significantly more effective as an antagonist at the AM1 receptor (pA2 = 7.40 ± 0.14; n = 3) than at the AM2 receptor (pA2 = 6.86 ± 0.09; n = 3; p < 0.05) (Hay et al., 2003). AM22–52 was also a weak antagonist at the CGRP receptor (Figs. 3 and 7; Table 3), having significantly lower affinity at this receptor compared with the AM1 receptor (p < 0.001) or the AM2 receptor (p < 0.01). AM23–52 was a lower affinity antagonist compared with AM22–53 at the AM1 receptor (p = 6.34 ± 0.17; n = 3; p < 0.01), the AM2 receptor (pA2 = 5.92 ± 0.04; n = 3; p < 0.001), and the CGRP receptor (pA2 < 5) (Table 3; Fig. 7). There was no significant difference in affinity between AM receptors. A similar pattern was observed for AM30–52, which was also a lower affinity antagonist at the AM1 receptor (pA2 = 6.04 ± 0.16; n = 3; p < 0.05), AM2 receptor (pA2 = 6.04 ± 0.16; n = 3; p < 0.05), and the CGRP receptor (pA2 < 5) compared with AM22–53 (Table 3; Fig. 7).

AM/αCGRP Chimeras. C1 (CGRP8–18AM33–52) was a moderate-affinity antagonist at both the AM1 receptor (pA2 = 7.41 ± 0.11; n = 3) and at the AM2 receptor (pA2 = 6.78 ± 0.21; n = 3) but showed increased affinity (pA2 = 6.97 ± 0.17; n = 4) for the CGRP receptor compared with AM22–52 (p < 0.01) (Table 3; Fig. 7). The difference between AM1 and AM2 receptors was not significant (p = 0.058). Introduction of a longer stretch of CGRP at the N terminus of AM22–52, generate C2 (CGRP8–14AM23–52) yielded a substantially higher affinity antagonist at the AM1 receptor (pA2 = 8.39 ± 0.16; n = 4), at the AM2 receptor (pA2 = 8.53 ± 0.17; n = 4), and at the CGRP receptor (pA2 = 8.25 ± 0.10; n = 4) compared with AM22–52 (p < 0.01, p < 0.001, and p < 0.01, respectively) (Table 3; Figs. 4 and 7). C3 (AM22–27CGRP14–23 AM38–52), in contrast, was a lower affinity antagonist com-

<table>
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<tr>
<th>AM9p–52</th>
<th>AM9p–52</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>C7</th>
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C/Math calculations data of CD spectra for AM, αCGRP, and AM2 were also (H11022 troscopy data were analyzed for secondary structure content using from unity. Unpaired nist tests were used to compare antagonist pEC50 values for each. A p

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Secondary Structure Composition. CD spectroscopy was used to compare secondary structure composition of peptides. The full-length peptides (AM, AM2, and αCGRP) were compared with each other and with their respective antagonist fragments (AM22–52 and CGRP8–37). C1 to C9 were compared with AM22–52. The three peptides AM, AM2, and αCGRP differed markedly in α-helix, β-sheet, and random coil content, which is not surprising given their lack of sequence homology. AM consists of 28% α-helix, AM2 shows 44% α-helix, and αCGRP contains 88% α-helix (Fig. 2; Table 1). Truncation of the N-terminal 21 residues of AM (AM22–52) did not change the secondary structure of the peptide; however, loss of the N-terminal seven residues of CGRP (CGRP8–37) caused a large change in secondary structure, with a major loss of α-helical content and a compensatory increase in β-sheet (Table 1). The similarities in the amino acid sequences of AM26–52, AM30–52, and C1 are reflected in
and the AM2 receptor (p<0.01; n=3) but showed similar affinity at the CGRP receptor (p=0.56; n=3). There was no significant difference in affinities between AM1, AM2, and CGRP receptors for peptides C1, C2, and C3. C4 (AM22–32CGRP19–23AM38–52) behaved in a similar manner to C3, acting as a lower affinity antagonist than AM22–52 at both the AM1 receptor (p=0.19; n=3; p<0.01) and the AM2 receptor (p=0.54; n=3; p=0.01) but showed similar affinity at the CGRP receptor (p=0.54; n=3) (Table 3; Fig. 7). This affinity was significantly lower than with AM22–52 (p<0.05). The final AM/αCGRP chimera tested in this study, C9 (CGRP8–29AM44–52), was equally effective as an antagonist at all three receptors (Table 3; Fig. 7). Its affinity was not different compared with AM22–52 at the AM1 or AM2 receptor.

Table 3
Summary of cAMP assay data

<table>
<thead>
<tr>
<th>Peptide</th>
<th>AM1</th>
<th>AM2</th>
<th>CGRP</th>
<th>Log Fold Selectivity, AM1 vs. AM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM22–52</td>
<td>7.40 ± 0.14 (3)</td>
<td>6.86 ± 0.09 (3)</td>
<td>5.96 ± 0.09 (3)</td>
<td>+0.54 (3)</td>
</tr>
<tr>
<td>AM22–32</td>
<td>6.34 ± 0.17 (3)</td>
<td>5.92 ± 0.04 (3)</td>
<td>&lt;5 (4)</td>
<td>+0.42 (4)</td>
</tr>
<tr>
<td>AM22–43</td>
<td>6.03 ± 0.35 (3)</td>
<td>6.04 ± 0.16 (3)</td>
<td>&lt;5 (4)</td>
<td>-0.01 (4)</td>
</tr>
<tr>
<td>C1</td>
<td>7.41 ± 0.11 (3)</td>
<td>6.78 ± 0.21 (3)</td>
<td>6.97 ± 0.17 (4)</td>
<td>+0.43 (4)</td>
</tr>
<tr>
<td>C2</td>
<td>8.39 ± 0.16 (4)</td>
<td>8.53 ± 0.17 (4)</td>
<td>8.25 ± 0.10 (4)</td>
<td>-0.14 (4)</td>
</tr>
<tr>
<td>C3</td>
<td>6.00 ± 0.36 (3)</td>
<td>5.94 ± 0.10 (3)</td>
<td>5.87 ± 0.13 (3)</td>
<td>+0.06 (3)</td>
</tr>
<tr>
<td>C4</td>
<td>6.16 ± 0.07 (3)</td>
<td>5.66 ± 0.24 (3)</td>
<td>5.76 ± 0.01 (4)</td>
<td>-0.5 (4)</td>
</tr>
<tr>
<td>C5</td>
<td>&lt;5 (3)</td>
<td>&lt;5 (3)</td>
<td>5.49 ± 0.06 (3)</td>
<td>+0.27 (3)</td>
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<tr>
<td>C6</td>
<td>8.37 ± 0.18 (3)</td>
<td>8.10 ± 0.08 (4)</td>
<td>8.21 ± 0.10 (3)</td>
<td>+0.27 (3)</td>
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<tr>
<td>C7</td>
<td>7.25 ± 0.13 (3)</td>
<td>7.81 ± 0.20 (4)</td>
<td>6.29 ± 0.19 (3)</td>
<td>-0.56 (3)</td>
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<tr>
<td>C8</td>
<td>6.84 ± 0.14 (3)</td>
<td>6.90 ± 0.15 (3)</td>
<td>6.36 ± 0.10 (3)</td>
<td>-0.06 (3)</td>
</tr>
<tr>
<td>C9</td>
<td>6.85 ± 0.21 (3)</td>
<td>7.02 ± 0.21 (3)</td>
<td>6.91 ± 0.06 (3)</td>
<td>-0.17 (3)</td>
</tr>
</tbody>
</table>

* C9 affinity was determined in COS-7 cells transfected with CGRP receptors, whereas the other antagonists were tested in SK-N-MCs, which endogenously express this receptor. AM22–52 affinity was not significantly different between these cells [SK-N-MC pA2 = 5.96 ± 0.09 (n = 3) vs. COS-7 pA2 = 5.54 ± 0.16 (n = 4); not significantly different by t test].

Fig. 3. cAMP responses to AM at AM receptors or αCGRP at the CGRP receptor with or without AM22–52 (10 μM). Data are representative of three to four independent experiments. Data points are mean ± S.E.M. of triplicate determinations.

Fig. 4. cAMP responses to AM at the AM receptors or αCGRP at the CGRP receptor with or without C2 (1 or 3 μM). Data are representative of three or four independent experiments. Data points are mean ± S.E.M. of triplicate determinations.
**AM/AM2 Chimeras.** Similar to C2, C6 (AM$_{22–28}$AM$_{23–30}$ AM$_{37–52}$) was a higher affinity antagonist than AM$_{22–52}$ at the AM$_1$ receptor (pEC$_{50}$ WT AM$_2$ = 8.37 ± 0.18; n = 3; p < 0.05), the AM$_2$ receptor (pEC$_{50}$ WT AM$_2$ = 8.10 ± 0.08; n = 4; p < 0.001), and the CGRP receptor (pEC$_{50}$ WT AM$_2$ = 8.21 ± 0.10; n = 3; p < 0.001) (Table 3; Figs. 5 and 7). However, there was no significant difference between the receptors. A different pattern was observed for C7 (AM$_{22–36}$AM$_{21–35}$AM$_{42–52}$), which was a moderate affinity antagonist at both the AM$_1$ receptor (pEC$_{50}$ WT AM$_2$ = 7.25 ± 0.13; n = 3) and a weak antagonist at the CGRP receptor (pEC$_{50}$ WT AM$_2$ = 6.29 ± 0.19; n = 3) but was a significantly higher affinity antagonist at the AM$_2$ receptor (pEC$_{50}$ WT AM$_2$ = 7.81 ± 0.20; n = 3) compared with AM$_{22–52}$ (p < 0.05) (Table 3; Figs. 6 and 7). The difference between the AM receptors was not significant but like AM$_{22–52}$, C7 was lower affinity at the CGRP receptor compared with the AM$_1$ (p < 0.05) or AM$_2$ (p < 0.01) receptors. C8 (AM$_{22–43}$AM$_{29–42}$AM$_{44–45}$AM$_{245–46}$AM$_{52}$) was a moderate-affinity antagonist at both the AM$_1$ receptor (pEC$_{50}$ WT AM$_2$ = 6.84 ± 0.14; n = 3) and the AM$_2$ receptor (pEC$_{50}$ WT AM$_2$ = 6.90 ± 0.15; n = 3) and was a weak antagonist (although higher affinity than AM$_{22–52}$) at the CGRP receptor (pEC$_{50}$ WT AM$_2$ = 6.36 ± 0.10; n = 3; p < 0.05) (Table 3; Fig. 7). Like C7, C8 had equal affinity at both AM receptors but had significantly lower affinity at the CGRP receptor compared with the AM$_1$ receptor (p < 0.05) or AM$_2$ receptor (p < 0.05).

**Contribution of Position 74 in RAMP1 and RAMP3 to AM$_{22–52}$ and C9 Affinity.** Given the known involvement of amino acid at position 74 in RAMP1 and RAMP3 in AM affinity, we sought to determine the impact of mutations at this position on antagonist affinity. COS-7 cells were transfected with the wild-type (WT) AM$_2$ receptor or E74W RAMP3 AM$_2$ receptors or WT CGRP receptors, alongside W74E RAMP1 CGRP receptors. The cells were stimulated with AM or αCGRP, respectively, in the absence or presence of antagonist. Consistent with previous studies, there was a decrease in AM potency at the E74W AM$_2$ receptor (pEC$_{50}$ WT AM$_2$ = 10.1 ± 0.10; n = 3) versus E74W AM$_2$ (9.29 ± 0.09; n = 3; p < 0.01 by t test) but no significant change in CGRP potency at the W74E CGRP receptor (pEC$_{50}$ WT = 9.90 ± 0.25; n = 3) versus W74E (9.99 ± 0.22; n = 3). At the E74W AM$_2$ receptor AM$_{22–52}$ affinity was also reduced (pEC$_{50}$ WT AM$_2$ = 6.70 ± 0.09; n = 5) versus E74W AM$_2$ (5.82 ± 0.10; n = 5; p < 0.001 by t test), with a reciprocal gain in affinity observed at the W74E CGRP receptor (pEC$_{50}$ WT CGRP 5.54 ± 0.16 (n = 4) versus W74E CGRP (6.26 ± 0.10; n = 4; p < 0.01 by t test) (Fig. 8). The lack of affinity of C5 for AM receptors suggested that the key region for AM receptor interactions was lost in this peptide, i.e., the extreme C terminus. Therefore, the peptide C9, which contains only this region, was used as a control. The results were equivalent to those obtained for AM$_{22–52}$, with a substantial loss of affinity at the E74W AM$_2$ receptor, compared with WT (pEC$_{50}$ WT AM$_2$ = 7.02 ± 0.21; n = 3) versus E74W AM$_2$ (6.17 ± 0.03; n = 3; p < 0.05 by t test) but a gain at the W74E CGRP receptor compared with WT (pEC$_{50}$ WT CGRP = 6.91 ± 0.06; n = 3) versus W74E CGRP (7.63 ± 0.09; n = 3; p < 0.01 by t test) (Fig. 9).

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**Fig. 5.** cAMP responses to AM at the AM receptors or αCGRP at the CGRP receptor with or without C6 (1 or 3 μM). Data are representative of three or four independent experiments. Data points are mean ± S.E.M. of triplicate determinations.

**Fig. 6.** cAMP responses to AM at the AM receptors or αCGRP at the CGRP receptor with or without C7 (10 μM). Data are representative of three or four independent experiments. Data points are mean ± S.E.M. of triplicate determinations.
Discussion

With a paucity of data on the structure-function of AM, the development of improved antagonists is challenging. Therefore, the aim of this study was to explore the importance of different regions of the AM peptide in interactions with AM1 and AM2 receptors for antagonist affinity. Accordingly, we generated a series of novel peptides, including AM fragments, AM/αCGRP chimeras, and AM/AM2 chimeras, based on the current antagonist AM22–52. These provide new information on determinants needed for affinity at AM and CGRP receptors.

Because there is no secondary structure information available for any form of AM, we compared the secondary structures of AM, AM2, and AM22–52 with that of αCGRP and the chimeras incorporating regions of the parent peptides using circular dichroism spectroscopy. Our analysis has been determined for peptides dissolved in 50% TFE. This is a commonly accepted way of inducing secondary structure in peptides, but it may not accurately represent their conformation when interacting with receptors. Regardless, it does allow comparisons to be made about the propensity of the different peptides to adopt a defined secondary structure.

The 88% α-helical content of αCGRP found in the current study is consistent with previous reports of 72% under the same conditions (Hubbard et al., 1991); there is also agreement that the N-terminal ring stabilizes the α-helix. Our analysis of AM and AM2 suggests that both are considerably different not only to αCGRP but also to each other. Our data suggest that removal of the N-terminal ring in AM has little effect on secondary structure; however further truncation of AM to yield AM26–52 and AM30–52 produced a substantial loss in α-helical content, compared with AM22–52. Therefore, residues 22 to 25 of AM may be important for stabilizing an α-helix. This is supported by a similar loss of α-helix in C1, which contains residues eight to 11 of αCGRP at its extreme N terminus. Replacement of residues 44 to 52 of AM with the corresponding residues of AM2 (C8) resulted in a somewhat reduced α-helical content and a substantial increase in β-sheet, suggesting that the correct sequence of the C-terminal residues of AM may be required for the formation or stabilization of an α-helical structure. It has been reported previously that α-helical content correlates with the affinity of CGRP antagonist fragments (Mimeault et al., 1992). However, for the peptides in this study, differences in affinity or selectivity of these peptides did not necessarily correlate with differences in secondary structural composition. Thus, it seems that secondary structure cannot, by itself, be used as a guide to activity.

The AM/αCGRP chimeras provide new information on the relative importance of the different parts of CGRP for binding to the CGRP receptor. Residues eight to 18 of CGRP form an α-helix (Breeze et al., 1991). The progressive introduction of this structure into AM with C1 and C2 produces corresponding increases in its affinity for the CGRP receptor, demonstrating that it is important for recognition of the CGRP receptor. However, for the peptides in this study, differences in affinity or selectivity of these peptides did not necessarily correlate with differences in secondary structural composition. Thus, it seems that secondary structure cannot, by itself, be used as a guide to activity.

The AM/αCGRP chimeras provide new information on the relative importance of the different parts of CGRP for binding to the CGRP receptor. Residues eight to 18 of CGRP form an α-helix (Breeze et al., 1991). The progressive introduction of this structure into AM with C1 and C2 produces corresponding increases in its affinity for the CGRP receptor, demonstrating that it is important for recognition of the CGRP receptor. However, C9 contains the CGRP sequence found in C2 plus additional CGRP sequence, but it was lower affinity than C2, demonstrating that the context of the sequence is crucial for the pharmacology demonstrated by any given peptide. It is interesting that the C terminus of CGRP (residues 30–37; C5) is not, by itself, able to enhance affinity at the CGRP receptor. Residues 28 to 37 of αCGRP can bind independently to the CGRP receptor but with low affinity (Poyner et al., 1998).

This study provides considerable information on regions needed for binding to AM1 and AM2 receptors. Progressive deletion of residues 22 to 30 from AM reduced affinity at both AM receptors. The corresponding region in CGRP (i.e., the

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**Fig. 7.** Summary of cAMP assay data for AM1 receptor (A), AM2 receptor (B), and CGRP receptor (C). Data are mean pA2 ± S.E.M. of three to five independent experiments performed in triplicate. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 versus AM22–52 by unpaired t test. Solid shaded symbols represent antagonists with higher affinity than AM22–52, gray shading indicates no change in affinity, and open symbols represent antagonists with decreased affinity compared with AM22–52.
residues directly after the ring structure) is also required for high-affinity binding (Mimeault et al., 1992). Residues 22 to 25 of AM seem to be more important than 26 to 29 because their removal resulted in a similar loss of affinity to removal of the residues 22 to 29. As with CGRP, this region, (i.e., AM_{22–29}) is not the sole determinant of AM affinity at either the AM_1 or AM_2 receptors. In C2, it has been entirely replaced by sequence from CGRP, but the resulting derivative has a higher affinity than AM_{22–52} and so residues 33 to 52, in the correct context, form an important binding epitope for AM receptors.

Progressive replacement of residues 28 to 37 of AM with the corresponding residues of CGRP (C3 and C4, respectively) resulted in decreased peptide affinity for both AM receptors but no change in affinity for the CGRP receptor. One significant difference between the peptides at this point in the sequence is the presence of charged residues in AM, suggesting that electrostatic interactions could help in stabilization of secondary structure or be involved in AM receptor recognition.

C5 consists of residues 22 to 43 of AM and the C terminus (residues 30–37) of CGRP. C5 had no affinity for either of the two AM receptors but retained weak affinity for the CGRP receptor. Because C5 had no measurable affinity at AM_1 or AM_2 receptors, we can speculate that the key AM-receptor binding region (i.e., extreme C terminus of AM) has been lost in this peptide chimera. This is consistent with other studies showing that correct C-terminal sequence in AM_{22–52} is required for binding to AM receptors (Eguchi et al., 1994). It is interesting that P49 is present in both AM and AM2 but not in other members of the calcitonin peptide family (also C5) and therefore may be an important residue for binding to AM receptors. The decreased length of C5 in the C-terminal region may also be significant. C9, which is essentially the reciprocal chimera to C5, containing residues eight to 29 of CGRP and 44 to 52 of AM, was AM_{22–52}-like in terms of affinity at AM_1 and AM_2 receptors. Given the potentially important role of the AM C terminus in binding, C9 provided a useful opportunity for further probing the interactions between AM and its receptors. We have established previously that mutations at position 74 of RAMP1 and -3 have significant effects on the affinity of full-length AM but not CGRP (Qi et al., 2008). These changes were also seen with C9, demonstrating that a locus contained somewhere in the final nine amino acids of AM is in proximity to position 74 of RAMP1 or -3.

The AM/CGRP chimeras show a range of preferential affinities for AM_1 versus AM_2 receptors; this is usually in favor of the AM_1 receptor (Table 3). There is some evidence that AM 26 to 29 and its surrounding residues is an important positive selector for AM_1 receptors. The peptides that show a preference for the AM_1 receptor (AM_{22–52}, AM_{26–52}, AM_{22–52},
C1, and C4) contain this region either entirely or abutting onto further AM sequence. However, the effect of this sequence seems to be context-dependent; it is present in C7 and C8 (and largely present in C6), but none show marked AM1 receptor selectivity. In C8, residues 44 to 52 of AM were replaced with the corresponding residues of AM2. The greatest pharmacological impact of these substitutions was a loss of affinity at AM1 receptors. It therefore seems likely that the C-terminal region of AM is more important for affinity at AM1 versus AM2 receptors and that this negates any selectivity effect of AM 26 to 52. The C terminus of AM22–52 has proline residues at positions 43 and 49, whereas C8 incorporates an additional proline at position 46. This additional proline may be important for changes in the C8 secondary structure compared with AM22–52 and loss of affinity at AM receptors.

C7, which replaces residues 37 to 41 of AM with the corresponding residues of AM2 (GRQDS) and lengthens the chimera, was similar to AM22–52 in terms of its pharmacology at AM1 and CGRP receptors. However, at the AM2 receptor it showed an ∼10-fold increase in affinity. This gain in affinity made it modestly AM2 receptor-selective. It still has residues 26 to 29, but it also has a five-amino acid substitution of AM22–52, which disrupts a sequence of alternating positive and negatively charged residues. Not only does this override any effects of AM 26 to 29 but also it seems to be a positive discriminator for AM2 receptors. This region therefore provides a target for future studies; it may be possible to generate an AM2 receptor-selective peptide antagonist.

Some common themes emerge from the study of these chimeras. Several distinct epitopes have been suggested to be important for receptor binding. For example, this study has confirmed the importance of the peptide C terminus in receptor interactions. However, these epitopes cannot be simply transferred from one peptide to another with easily interpretable results; their effects depend on the rest of the sequence. It seems that changes some distance from the epitope can influence peptide behavior. This can best be understood in terms of the peptides making multiple contacts with the various CL/RAMP complexes. Furthermore, structural studies of ligand association with other family B G protein-coupled receptors reveal a broadly conserved mechanism of binding. The peptides sit in a broad groove and pack against an N-terminal helix, which is part of the receptor. Clearly, this mode of binding must be perturbed by the presence of RAMPs, but if the groove is maintained in some form, it may be able to accommodate a variety of shapes of peptides. Thus, the chimeras may be able to interact with each receptor in different ways.

The work has revealed key regions, such as GRQDS (in C7), that may be targeted in future studies, for example by alanine scans. We also determined that the C-terminal nine amino acids of AM are likely to be involved in interactions with Glu74 in RAMP3. Understanding the structure-activity

![Fig. 9.](https://example.com/fig9.png)
relationships of AM and related peptides is crucial for generating AM1 or AM2 receptor-selective antagonists.

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References


Address correspondence to: Dr. Debbie L. Hay, School of Biological Sciences, Thomas Bldg., Level 2, 3A Symonds St., The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand. E-mail: dl.hay@ auckland.ac.nz