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The selectivity and structural determinants of peptide antagonists at the CGRP receptor of rat, L6 myocytes

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1 Potency orders were determined for a series of agonists and antagonists on the calcitonin gene-related peptide (CGRP) receptor of rat L6 myocytes. The agents tested were all shown to have been active against CGRP, amylin or adrenomedullin receptors.

2 AC187 had a pIC_{50} of 6.8 ± 0.10, making it 14 fold less potent as an antagonist than $CGRP_{8-37}$ (pIC_{50}, 7.95 ± 0.14). $Amyline_{8-37}$ was equipotent to AC187 (pIC_{50}, 6.6 ± 0.16) and $CGRP_{19-37}$ was 3 fold less potent than either (pIC_{50}, 6.1 ± 0.24).

3 [Ala^{11}]-$CGRP_{8-37}$ was 6 fold less potent than $CGRP_{8-37}$, (pIC_{50}, 7.13 ± 0.14), whereas [Ala^{18}]-$CGRP_{8-37}$ was approximately equipotent to $CGRP_{8-37}$ (pIC_{50}, 7.52 ± 0.15). However, [Ala^{11},Ala^{18}]-$CGRP_{8-37}$ was over 300 fold less potent than $CGRP_{8-37}$ (pIC_{50}, 5.30 ± 0.04).

4 [Tyr^{0}]-$CGRP_{28-37}$, $amylin_{19-37}$ and $adrenomedullin_{22-52}$ were inactive as antagonists at concentrations of up to 1 μM.

5 Biotinyl-human α-CGRP was 150 fold less potent than human α-CGRP itself (EC_{50} values of 48 ± 17 nM and 0.31 ± 0.13 nM, respectively). At 1 μM, [Cys(acetomethoxy)^{2,7}]-CGRP was inactive as an agonist.

6 These results confirm a role for Arg^{11} in maintaining the high affinity binding of $CGRP_{8-37}$. Arg^{18} is of less direct significance for high affinity binding, but it may be important in maintaining the amphipathic nature of CGRP and its analogues.

Keywords: Calcitonin-gene related peptide; amylin antagonists; CGRP antagonists; CGRP receptors

Introduction

Calcitonin gene-related peptide (CGRP), amylin and adrenomedullin are related peptides (Poyner, 1995). CGRP is an abundant, 37 amino acid neuropeptide (Poyner, 1992) which, amongst other actions, inhibits insulin-stimulated glycogen synthesis in rat skeletal muscle (Leighton & Cooper, 1988). Amylin is also a 37 amino acid peptide with 47% sequence identity with CGRP. It has similar metabolic actions to CGRP, and it has been suggested that it might play a physiological role in regulating glucose metabolism in muscle (Rink *et al*., 1993). Adrenomedullin is a 52 amino acid peptide which can also activate CGRP receptors (e.g. Zimmerman *et al*., 1995).

It has been proposed that CGRP receptors can be divided into two classes; $CGRP_1$, which have a high affinity for the CGRP antagonist $CGRP_{8-37}$ and $CGRP_2$ which have a lower affinity for $CGRP_{8-37}$ but which can be activated by the linear CGRP analogue [Cys(acetomethoxy)^{2,7}]-CGRP (cysACM-CGRP) (Dennis *et al*., 1989; 1990). Although this classification has not met with universal support (e.g. Longmore *et al*., 1994), it does appear that especially in the rat, there are cells and tissues which express receptors with a particularly high affinity for $CGRP_{8-37}$ (Poyner, 1995). These include L6 skeletal myocytes where $CGRP_{8-37}$ has a pA_2 of 8.3 (Poyner *et al*., 1992), over an order of magnitude greater than any value found for the rat vas deferens where $CGRP_2$ receptors have been defined. Amylin appears to have its own receptor on rat skeletal muscle which can be antagonized by the salmon calcitonin derivative AC187 (acetyl-[Asn^{30},Tyr^{32}]salmon $calcitonin_{8-32}$) and also by $amylin_{8-37}$ (Deems *et al*., 1991; Beaumont *et al*., 1995). Although they can interact at CGRP receptors, the specificity of these compounds has not been studied in any detail (Deems *et al*., 1991; Beaumont *et al*., 1995; Tomlinson & Poyner, 1996). $Adrenomedullin_{22-52}$ has been shown to antagonize at least some actions of adrenomedullin, but its specificity is also largely unknown (Eguchi *et al*., 1994; Zimmerman *et al*., 1996).

L6 skeletal myocytes are a useful model system with which to study CGRP receptors. These express $CGRP_1$-like receptors which increase adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and modulate glucose metabolism, but lack amylin receptors (Kreutter *et al*., 1989; Poyner *et al*., 1992; Pittner *et al*., 1996). Although they have been found to have binding sites for adrenomedullin, these are not linked to the production of cyclic AMP (Coppock *et al*., 1996). Thus it is possible to examine the effects of putative amylin, adrenomedullin and CGRP antagonists on the CGRP receptors in isolation, to determine their selectivity. This is particularly important for AC187; although a K_d for this compound against CGRP receptors has been determined by radioligand binding (Beaumont *et al*., 1995), this technique does not always give a good indication of pA_2 values for CGRP antagonists (Dennis *et al*., 1990). In addition, in the light of its high affinity for $CGRP_{8-37}$, it is of interest to examine the structure-activity relationship for this CGRP receptor. Accordingly, these two issues have been investigated in this study.

Part of this work has been previously presented in abstract form (Howitt & Poyner, 1996).

Methods

Cell culture and assay of CGRP-stimulated cyclic AMP production were as described by Poyner *et al*. (1992). Briefly, cells were grown to confluence in 24 well plates and maintained typically for a further 5 days. They were then pretreated with antagonists for 5 min, before addition of 10 nM human α-CGRP for 10 min (this concentration of CGRP is just maximal, see Poyner *et al*., 1992). Medium was replaced with 0.5 ml of 20 mM Tris, 5 mM EDTA and incubations terminated by boiling for 5 min. The cyclic AMP was measured by a radio-receptor assay with [^{3}H]-cyclic AMP and the cyclic AMP binding subunit of protein kinase A. Synthesis of peptides

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(AC187, fragments of CGRP, adrenomedullin and amylin) was as described previously (Tomlinson & Poyner, 1996). Briefly, peptides were made on an Applied Biosystems 430A peptide synthesizer with FMOC chemistry, followed by cleavage in trifluoroacetic acid and purification by high performance liquid chromatography (h.p.l.c.).

Data evaluation

Individual dose-response curves were fitted with EBDA-Ligand to obtain EC_{50}/IC_{50} values. Negative logarithms of these have been taken so they are expressed as pIC_{50} values. Throughout the text, values are quoted as means ± s.e. Statistical analysis was by one-way ANOVA followed by Dunnett's test (multiple comparisons) or by Student's *t* test as appropriate, accepting significance at $P<0.05$.

Drugs

Peptides were obtained from Calbiochem (human α-CGRP) or Peninsula (CysACM-CGRP, biotinyl human α-CGRP). Other reagents were obtained as described previously (Poyner *et al.*, 1992).

Results

Inhibition of CGRP-stimulated cyclic AMP production by peptide fragments

Consistent with previous results, $CGRP_{8-37}$ produced a potent, dose-dependent inhibition of the cyclic AMP production caused by 10 nM human α-CGRP, with a pIC_{50} of 7.95 ± 0.14 ($n=8$, Figure 1). As can be seen from Figure 1a, a variety of other peptide fragments were also able to inhibit the response to 10 nM human α-CGRP, with pIC_{50} values as follows; AC187, 6.8 ± 0.10 ($n=5$), $amylin_{8-37}$, 6.6 ± 0.16 ($n=5$) and $CGRP_{19-37}$, 6.1 ± 0.24 ($n=6$). $[Tyr^0]$-$CGRP_{28-37}$ and $amylin_{19-37}$ were inactive at 1 μM (reductions in 10 nM CGRP-stimulated cyclic AMP production of 22 ± 10%, $n=5$ and 17 ± 10%, $n=5$, respectively). $Adrenomedullin_{22-52}$ 1 μM also failed to inhibit CGRP-stimulated cyclic AMP production, and at 10 μM it increased cyclic AMP content to 102 ± 8 pmol per 10^6 cells (68% of the stimulation seen with 10 nM CGRP). This effect was not via a CGRP receptor, since it was not blocked by 1 μM $CGRP_{8-37}$ (response 103 ± 13% of that seen in the absence of $CGRP_{8-37}$, $n=3$).

As $CGRP_{8-37}$, $amylin_{8-37}$ and AC187 were all reasonably good antagonists, their structures were compared in an attempt to identify common features. All have basic groups at sites which correspond to positions 11 and 18 of CGRP, and it was hypothesized that these might be important for binding to the CGRP receptor found on L6 cells. $CGRP_{8-37}$ derivatives were prepared with one or both of these residues replaced with alanines. $[Ala^{11}]$-$CGRP_{8-37}$ was approximately 6 fold less potent than $CGRP_{8-37}$ ($pIC_{50}=7.13\pm0.14$, $n=4$, Figure 1b); a significant difference ($P<0.05$). $[Ala^{18}]$-$CGRP_{8-37}$ was under 3 fold less potent than $CGRP_{8-37}$ ($pIC_{50}=7.52\pm0.15$, $n=3$, Figure 1b). This was not significant. However $[Ala^{11},Ala^{18}]$-$CGRP_{8-37}$ was over 300 fold less potent than $CGRP_{8-37}$ ($pIC_{50}=5.30\pm0.04$, $n=3$, Figure 1b).

Schild analysis for AC187

To investigate the mechanism of action of AC187 at the CGRP receptor, a Schild plot was constructed based on shifts of the CGRP dose-response curve produced by concentrations of the antagonist from 100 nM to 3 μM (Figure 2). The resulting line had a slope of 0.73 ± 0.10 ($n=4$), not significantly different from unity (95% confidence limits 0.30 – 1.16), consistent with a competitive interaction. By averaging the four dose shifts in Figure 2, an apparent pA_2 of 6.91 ± 0.06 ($n=4$) was estimated,

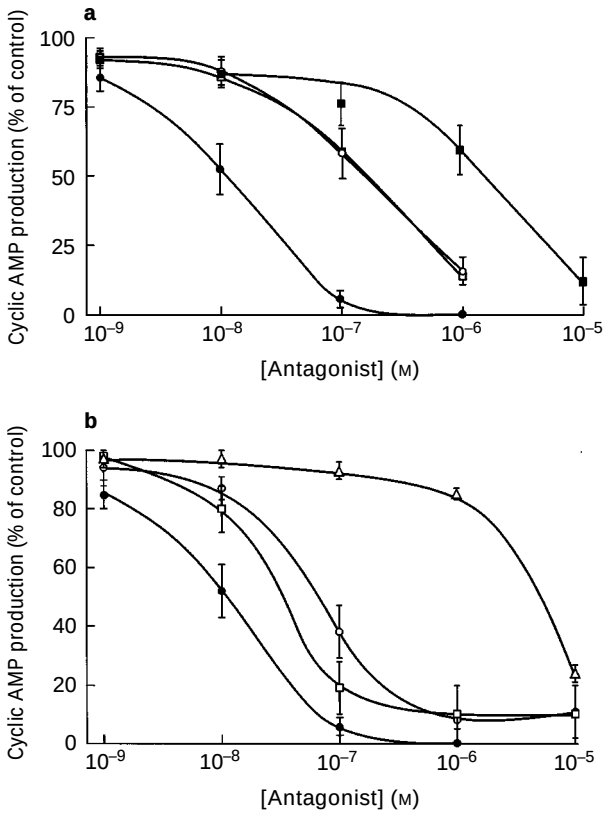

Figure 1 (a) Inhibition of 10 nM CGRP-stimulated cyclic AMP production by (●) $CGRP_{8-37}$, (○) AC187, (□) $amylin_{8-37}$ and (■) $CGRP_{19-37}$. (b) Inhibition of the above response by (●) $CGRP_{8-37}$, (○) $[Ala^{11}]$-$CGRP_{8-37}$, (□) $[Ala^{18}]$-$CGRP_{8-37}$ and (△) $[Ala^{11},Ala^{18}]$-$CGRP_{8-37}$. Cyclic AMP accumulation was measured following 10 min exposure to CGRP, and at 100% the absolute accumulation was 150 pmol per 10^6 cells. Each point represents the mean of 3 to 5 separate determinations; vertical lines show s.e.mean.

in fair agreement with the results from inhibition of CGRP-stimulated adenylate cyclase production.

Agonist actions at the receptor

Biotinyl-human α-CGRP was 150 fold less potent than human α-CGRP, with the EC_{50} increasing from 0.31 ± 0.13 nM to 48 ± 17 nM. The putative $CGRP_1$-selective agonist, CysACM-CGRP was inactive at concentrations of up to 1 μM.

Discussion

These present results give new information on the pharmacology of the $CGRP_1$-like receptor on rat, L6 cells and provide a detailed account of the antagonist action of several CGRP, amylin and adrenomedullin analogues. The results of this study have implications for the development of selective antagonists for amylin and adrenomedullin receptors, for the structure-activity relationship at CGRP receptors, and for the classification of CGRP receptor subtypes.

The inability of CysACM-CGRP to activate the CGRP receptor on these cells confirms its pharmacology as $CGRP_1$-like. Little has been published on the pharmacology of biotinylated CGRP, but this modification is poorly tolerated, either because of the size of the biotinyl group, or the removal of the N-terminal positive charge on CGRP.

$Amylin_{8-37}$ and AC187 have been used in the study of amylin receptors. Beaumont *et al.* (1995) have produced data for AC187 consistent with it having a pA_2 of 8.3 against amylin on rat soleus. They did not determine a pA_2 for it against

CGRP on the CGRP receptor of L6 cells, although they did establish that it had a p*K*d of 7.1 in a radioligand binding assay. Thus, there is good agreement between the p*K*d of AC187 from binding studies and the apparent pA_2 of 6.9 found in this study. This confirms that AC187 has about 30 fold selectivity for amylin over CGRP receptors in rat skeletal muscle. Provided that AC187 is used with care, it would appear to have useful selectivity for amylin receptors. The data in this study is consistent with AC187 acting as a simple competitive antagonist at the L6 CGRP receptor, although some caution is required on this point due to the rather wide confidence limits associated with the slope of the Schild plot. $Amylin_{8-37}$ is not so well characterized as AC187; it has been found that, at 1 μM, it will cause about 50% blockade of the inhibition of glycogen synthesis in response to 10 nM amylin in the isolated soleus muscle (Deems *et al.*, 1991). As amylin has been shown to have an EC_{50} of about 5 nM against inhibition of glycogen synthesis (Pittner *et al.*, 1996), this is consistent with $amylin_{8-37}$ having an apparent pA_2 of about 7 on amylin receptors. In the present study $amylin_{8-37}$ was roughly equipotent with AC187 i.e. apparent pA_2 of about 6.9. Given the crudity of the above calculations, it would be unwise to read too much into these figures, but they do suggest that $amylin_{8-37}$ is certainly not more selective than AC187, and might be considerably less so. The putative adrenomedullin antagonist, $adrenomedullin_{22-52}$ is also poorly defined, but as it was without effect on the CGRP receptor at concentrations of up to 10 μM, cross-reactivity at this site seems unlikely to be a serious problem.

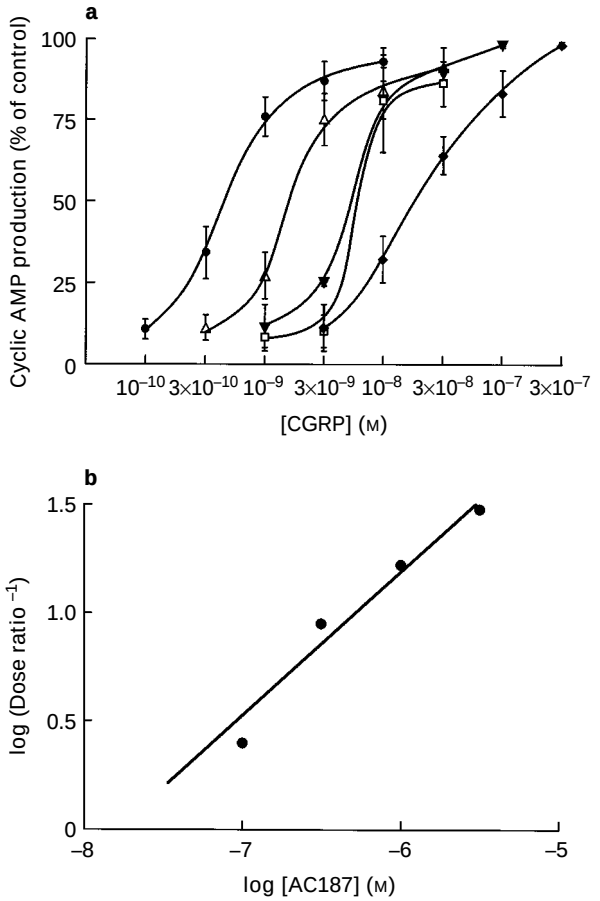

Figure 2 (a) Inhibition of CGRP-stimulated cyclic AMP production by AC187 (△) 100 nM, (▼) 300 nM, (□) 1 μM and (◆) 3 μM; (●) control responses in the absence of AC187. (b) Schild plot of the above data. Each point represents the mean of 3 to 5 determinations; vertical lines show s.e.mean.

AC187 and $amylin_{8-37}$ have relatively high affinities for the L6 CGRP receptor; greater than that of $CGRP_{19-37}$. This is surprising, particularly for AC187 which is a salmon calcitonin derivative with only 34% homology to human α-$CGRP_{8-37}$. The area of closest homology is at the C-terminus, where 5/8 residues are either homologous or identical (Figure 3a). However, these residues are unlikely by themselves to account for the relatively high affinity of AC187, since they are also found in the fragment [Tyr^0]-$CGRP_{28-37}$ which has a very low affinity against this receptor. Boulanger *et al.* (1996) concluded that although the C-terminus is essential for binding, it is necessary for it to be involved in interactions with other regions of the molecule to maintain it in an appropriate conformation. AC187, $CGRP_{8-37}$ and $amylin_{8-37}$ all have a region of potential amphipathic α-helix at their N-terminus (Epand *et al.*, 1985; Lynch & Kaiser, 1988), which also contains two conserved basic residues corresponding to Arg^{11} and Arg^{18} of human α-CGRP (Figure 3b). It has been concluded that this structure is critical for high affinity interactions with the CGRP receptor (Mimeault *et al.*, 1992). Its presence has been inferred from circular dichroism measurements made in hydrophobic solvent mixtures (Hubbard *et al.*, 1991), and substitution of Arg^{11} by alanine reduces helical content and also antagonist potency at the guinea-pig $CGRP_1$ receptor (Mimeault *et al.*, 1992). However, two-dimensional nuclear magnetic resonance studies have suggested that this region is largely a random coil (Boulanger *et al.*, 1995), with no obvious relationship between the aqueous solution structure and antagonist potency in a variety of alanine-substituted analogues (Boulanger *et al.*, 1996). Thus it seems both that the conformations favoured by $CGRP_{8-37}$ and the forces promoting

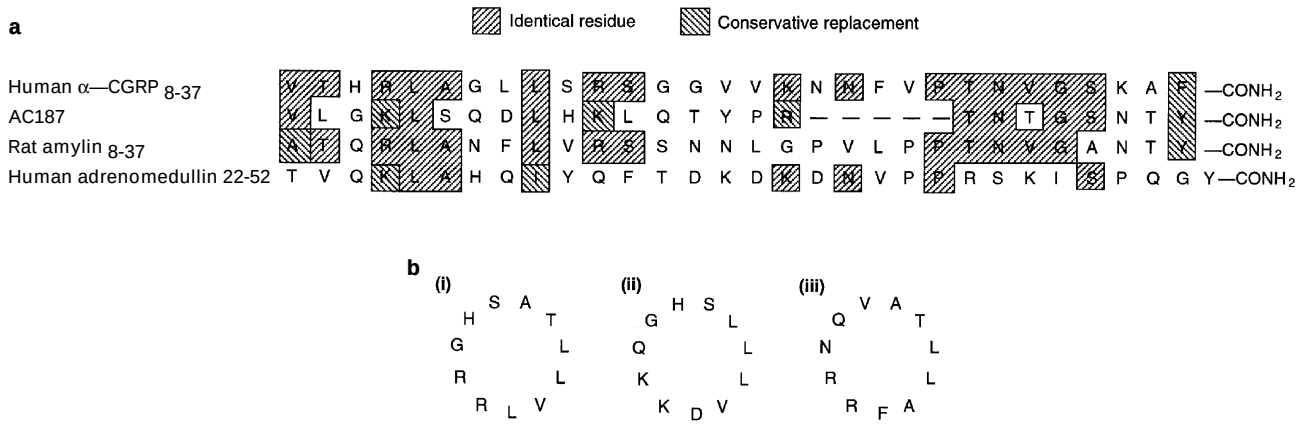

Figure 3 (a) Comparison of the structure of $CGRP_{8-37}$ with AC187, $amylin_{8-37}$ and $adrenomedullin_{22-52}$. (b) Helical wheel projections of residues 8–18 of (i) $CGRP_{8-37}$, (ii) AC187 and (iii) $amylin_{8-37}$. These regions all show α-helical forming potential, based on secondary structure predictions (data not shown).

these conformations are not fully understood. This complicates the theoretical interpretation of the results found in the present study. However, the data for [Ala^{11}]-CGRP$_{8-37}$ confirm a role for Arg^{11} in binding to CGRP$_1$-like receptors, regardless of how exactly it acts. It is worth noting that the effect of its substitution was rather small. Arg^{18} is of less importance in sustaining high affinity binding. (Recently Boulanger *et al.* (1996) have obtained similar data for Ser^{17} and Gly^{20}). However, the doubly substituted analogue [Ala^{11},Ala^{18}]-CGRP$_{8-37}$ was 300 times less potent than CGRP$_{8-37}$. Perhaps the most obvious effect of the double substitution compared to the single substitution is that the amphiphatic nature of this region of the peptide will be largely lost. This would support the view that whatever structure the peptide adopts in aqueous solution, an amphipathic helical structure is important for binding to the receptor. It is possible that the most important role of Arg^{11} and Arg^{18} in high affinity interactions is in maintaining amphipathicity. It remains to be established whether this structure interacts directly with the receptor, or whether it interacts with other regions of the peptide that are actually involved in receptor contacts.

The rat L6 CGRP receptor can be compared with other receptors which have a high affinity for CGRP$_{8-37}$. In the rat, there are a number of tissues which show a comparable pA$_2$ of about 8.0 (see Poyner, 1995 for review), but these have not been examined in detail with other antagonists. More information is available on the prototypic CGRP$_1$ receptors of the guinea-pig atrium and ileum (Dennis *et al.*, 1990; Mimeault *et al.*, 1992; Tomlinson & Poyner, 1995). These both have a pA$_2$ for CGRP$_{8-37}$ of about 7.0. Against human α-CGRP on the ileum, AC187 and CGRP$_{19-37}$ were both three fold less potent than CGRP$_{8-37}$, with [Tyr^0]-CGRP$_{28-37}$ twenty fold less potent. On the atrium [Ala^{11}]-CGRP$_{8-37}$ was about 4 fold less potent than CGRP$_{8-37}$ itself. CysACM-CGRP was inactive at concentrations up to 1 μM on the ileum (A.E. Tomlinson & D.R. Poyner, unpublished results). These results are broadly similar to the pharmacology of the rat L6 CGRP receptor described in this present study, although there are differences in the details (e.g. the relative affinities of AC187 and CGRP$_{19-37}$). Given the absence of both sequence data on the two receptor types and a wider range of structurally diverse antagonists, it would be unwise to draw too many conclusions as to how they may be related. However, the data available are consistent with them being regarded as species homologues (Poyner, 1995).

In conclusion, this study provides new information on the pharmacology of the rat L6 CGRP receptor. For CGRP$_{8-37}$, Arg^{11} plays a role in high affinity binding to this receptor, but together with Arg^{18}, its chief importance is probably in maintaining the peptide's amphipathic nature. The receptor is clearly distinct from the receptor through which amylin exerts its metabolic actions on rat soleus muscle. It is similar, but not identical to the CGRP$_1$ receptor found on the guinea-pig ileum, emphasizing the importance of recognizing species variation when considering the classification of CGRP receptors.

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