On the role of the C-terminus of α-calcitonin-gene-related peptide (αCGRP)

The structure of des-phenylalaninamide[37]-αCGRP and its interaction with the CGRP receptor

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α-Calcitonin-gene-related peptide (αCGRP) lacking its C-terminal phenylalaninamide residue was found not to bind to its receptor as did full-length (amidated) αCGRP. Investigation of the structure of these peptides by c.d. and n.m.r. revealed no significant difference, so it seemed that the effect of deleting the C-terminal phenylalaninamide on the biological activity of αCGRP was not by disruption of the peptide's structure. Thus the C-terminal phenylalaninamide is an important factor in this ligand–receptor interaction, and the group itself may interact directly with the receptor.

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a 37-amino-acid-long product of alternative calcitonin mRNA splicing (Amara et al., 1982). It can affect various biological processes. It is, for instance, a potent vasodilator with positive chronotropic and inotropic effects in atrial tissues (Brain et al., 1985; Yamaguchi et al., 1988), and it can inhibit insulin-stimulated glycogen synthesis in skeletal muscle (Leighton et al., 1989). CGRP exerts its effect by interaction with specific receptors of which there are at least two types, 1 and 2. These two can be differentiated on the basis of sensitivity to antagonism (of type 1 receptors preferentially) by a CGRP fragment including residues 12–37 (CGRP 12–37), or 8–37 (CGRP 8–37), and by agonism (of type 2 receptors preferentially) by the linear analogue [acetamidomethylcysteine[2,7]]CGRP (Dennis et al., 1989; Donoso et al., 1990). Type 2 receptors have only been recognized in the rat vas deferens to date (Dennis et al., 1989) but type 1 receptors possibly mediate the cardiovascular effects of CGRP (Dennis et al., 1989; Donoso et al., 1990) and its effects on skeletal-muscle cells, since this is antagonized by CGRP 8–37 (Zhu et al., 1991; Poyner et al., 1992).

In man, CGRP exists as two species, α and β, but the biological significance of this diversity is unclear. The amino acid sequences of α-CGRP and β-CGRP differ by only three amino acids (Steenburgh et al., 1985). The CGRP sequence has been conserved during evolution such that rat αCGRP (Amara et al., 1985) and chicken CGRP (Minvielle et al., 1987) each differ from human αCGRP in only four places. Each is thought to contain a disulphide bond between cysteine residues at positions 2 and 7, and each is thought to carry an α-amide group at its C-terminus.

Studies of the structure of CGRP shows the presence of the disulphide bonded loop near the N-terminus, and suggest that this is immediately adjacent to an α-helical segment in the region from residue 8 up to about 18. The structure of the remaining, C-terminal, segment of the CGRP molecule is not well defined, though modelling suggests a turn immediately after the α-helix in the region of residues 19–23 and possibly another in the region 29–34 (Lynch and Kaiser, 1988; Breeze et al., 1991; Hubbard et al., 1991). A study of an N-terminally modified fragment of CGRP (encompassing the C-terminal residues 19–37), by Sagoo et al. (1991) has also suggested the possibility of a loop involving the C-terminus interacting with residue 30.

Which of the structural features of CGRP determine the interaction of the peptide with its specific receptor(s) is uncertain. For type 1 receptors it seems that an intact disulphide bond is required for biological activity, though a linear CGRP analogue lacking the disulphide is active on type 2 receptor(s) (Dennis et al., 1989). N-terminal fragments (with intact disulphide) show agonism (Maggi et al., 1990) [or, in another report, competitive antagonism (Dennis et al., 1989)], but these activities are much reduced compared with intact CGRP. Seemingly these fragments have lost most of their ability to bind to the receptor. The major determinants for binding reside in the region of residues 8–37-NH_2, which constitute an antagonist (Dennis et al., 1989; Donoso et al. 1990). Changes in the helical region have been found to alter biological activity (Lynch and Kaiser, 1988) and could be analogous to the situation in which the α-helix content of salmon calcitonin seems to be an important property for its binding to receptors on rat renal membranes (though not brain membranes) (Twery et al., 1988). There could be other determinants nearer the C-terminus, however, since inactivating anti-CGRP antibodies map functionally to the C-terminal ten residues (Andrew et al., 1990). Again, in the peptide amylin, of about 50% sequence identity with CGRP but of somewhat different structure (Hubbard et al., 1991), the presence of the C-terminal amide group is necessary for biological activity (Roberts et al., 1989). In order to determine the role of the C-terminus of CGRP we have studied the effects of deleting the C-terminal amino acid (phenylalanine) and amide, and report that this section of the molecule has a significant role in interaction with receptor(s).

METHODS

Cell culture

L6 myocytes (European Culture Collection) were grown in Dulbecco's modified eagle medium (DMEM) with 10% foetal-calf serum. For the purpose of bioassay of αCGRP and des-

Abbreviations used: αCGRP, α-calcitonin-gene-related peptide; desF-NH_2-αCGRP, des-phenylalaninamide[37]-αCGRP (αCGRP residues 1–36): BK, bradykinin; COSY, two dimensional correlation spectroscopy; n.O.e., (two-dimensional) nuclear Overhauser effect; NOESY, two-dimensional nuclear-Overhauser-enhancement spectroscopy; ROESY, rotating-frame Overhauser-enhancement spectroscopy; TOCSY, totally correlated spectroscopy; TFE, trifluoroethanol; TMS, tetramethylsilane; DMEM, Dulbecco's modified Eagle medium.

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Table 1 Relative increase in cyclic AMP concentration in intact L6 myocytes incubated with peptides

Each assay result is the mean for three experiments (with S.D.) presented relative to the effect of 10^{-8} M αCGRP. αCGRP at 10^{-8} M caused maximal elevation of [cyclic AMP] in these cells, giving an approx. 20-fold increase to approx. 200 pmol/10^6 cells (Poyner et al., 1992).

[Peptide] (M)			Relative cyclic AMP accumulation (%)
αCGRP	desF-NH_2-αCGRP	BK	
10^{-8}	0	0	100±3
0	10^{-8}	0	10±12
0	10^{-7}	0	8±10
0	10^{-6}	0	11±20
0	0	10^{-7}	16±4
10^{-8}	10^{-8}	0	87±11
10^{-8}	10^{-7}	0	91±12
10^{-8}	10^{-6}	0	97±13

phenylalaninamide(desF-NH_2)-αCGRP, cells were grown to confluence in 24-well plates.

Bioassay (cyclic AMP accumulation)

The bioassay was as described by Poyner et al. (1992), as follows. Cells and peptide were incubated together in DMEM/10% foetal-calf serum (500 μl per well) with gentle shaking for 5 min at 37 °C. Incubation was terminated by placing the 24-well plate on ice and replacing the medium with ice-cold buffer [20 mM Tris/HCl (pH 7.7)/5 mM EDTA]. The plate was then stood in boiling water for 5 min, then cooled on ice for 15 min. The stimulation of adenylate cyclase caused by any action of αCGRP (Bachem) or desF-NH_2-α-CGRP (Bachem) was quantified by assay of cyclic AMP, essentially as described by Gilman (1970), as follows: 50 μl of each incubation mixture was mixed with 50 μl of cyclic [^{3}H]AMP (Amersham) at 20 nM in 5 mM EDTA and with 100 μl of a solution of cyclic AMP-dependent protein kinase (Sigma) at 0.1 mg·ml^{-1} in 0.5 mM sodium citrate (pH 6.5)/1 mM dithiothreitol/2.5 mM EDTA. The mixture was incubated at 4 °C for 18 h. Then 100 μl of a suspension of activated charcoal (from BDH) at 5% (w/v) in 20 mM Tris/HCl (pH 7.7)/5 mM EDTA/0.2% (w/v) BSA mixed with each assay sample and centrifuged to pellet the charcoal, protein and protein–bound cyclic [^{3}H]AMP. Cyclic [^{3}H]AMP remaining in solution, a measure of competition for binding to protein kinase by cyclic AMP in assayed sample, was quantified by liquid-scintillation counting. Bradykinin (BK; Sigma) was used as a control peptide ligand. For study of antagonism by desF-NH_2-αCGRP, it was incubated together with αCGRP on the cells and the assay otherwise carried out as described above.

C.d. spectroscopy

C.d. spectra of CGRP and desF-NH_2-αCGRP were recorded over the range 250 to 190 nm using a JASCO J600 spectropolarimeter. The concentrations of peptides were in the range of 0.06–0.08 mg/ml, and the path length was 1 mm. Samples were dissolved in 20 mM sodium phosphate, pH 7.0, and the effects of including trifluoroethanol (TFE) (50%, v/v) or SDS (0.4%, w/v), and of varying the temperature over the range 20–37 °C, were monitored. The concentrations of peptides and the secondary-structure contents were determined as described previously (Hubbard et al., 1991), using data at 0.2 nm intervals over the range 240–190 nm.

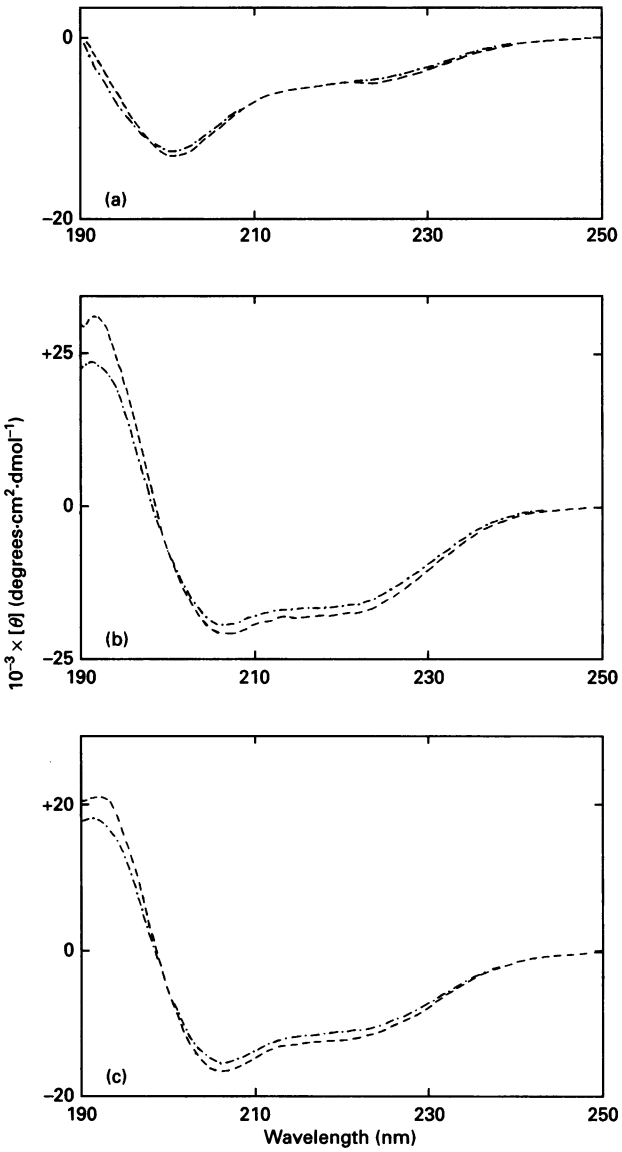

Figure 1 Far-u.v. c.d. spectra of αCGRP (----) and desF-NH_2-αCGRP (–·–·)

Spectra were recorded at 20 °C in a cell of path length 1 mm. (**a**) Spectra in buffer (20 mM sodium phosphate, pH 7.0); (**b**) spectra in buffer with 50% (v/v) TFE; (**c**) spectra in buffer with 0.4% (w/v) SDS.

N.m.r. spectroscopy

Human αCGRP or desF-NH_2-αCGRP (CGRP 1–36) was dissolved at 4 mM in water (containing 10% ^2H_2O) and 2 mM in 50% (v/v) trifluoroethanol/water mixtures and adjusted to a pH of between 3.7 and 3.8 (uncorrected for isotope effects) using a glass pH electrode. ^{1}H-n.m.r. spectra were recorded at 27 °C on either the home-built 500 MHz spectrometer of the Oxford Centre for Molecular Science (comprising an Oxford Instruments magnet and a GE/Nicolet 1280 computer) or a Bruker AMX series 500 MHz spectrometer.

Phase-sensitive two-dimensional preTOCSY COSY (pre-totally correlated spectroscopy) and preTOCSY nuclear-Overhauser-enhancement spectroscopy (NOESY) (mixing time 200 ms) spectra (Otting and Wuthrich, 1987) were obtained by using the method of States et al. (1982), using a data matrix of either 256 or 512 complex points by 2048. Typically 32 scans

Table 2 Structure analysis of far-u.v. c.d. spectra of CGRP and desF-NH_2-αCGRP in 20 mM sodium phosphate, pH 7.0 ('buffer')

Values are given as means ± S.E.M., derived by applying the CONTIN procedure (Provencher and Glöckner, 1981) to the spectra averaged from three scans of each sample.

Sample	Temp. (°C)	Conditions	Structure... Proportion (%) α-Helix	β-Sheet	Remainder
CGRP	20	Buffer	11 ± 1	36 ± 1	53 ± 1
		50% TFE	59 ± 1	41 ± 1	0
		0.4% SDS	35 ± 2	44 ± 2	21 ± 4
	37	Buffer	9 ± 1	35 ± 1	56 ± 1
		50% TFE	46 ± 2	24 ± 1	30 ± 3
		0.4% SDS	33 ± 1	37 ± 1	30 ± 2
desF-NH_2-αCGRP	20	Buffer	7 ± 2	25 ± 2	68 ± 3
		50% TFE	51 ± 2	31 ± 2	18 ± 2
		0.4% SDS	37 ± 1	41 ± 1	22 ± 2
	37	Buffer	13 ± 1	36 ± 1	51 ± 2
		50% TFE	49 ± 1	34 ± 1	17 ± 2
		0.4% SDS	34 ± 1	46 ± 1	20 ± 2

were averaged for each t_1 increment. The residual solvent signal was suppressed using presaturation. Typical mixing times for the preTOCSY spin-lock period was 25 ms. Data were processed on a Nicolet data station. Two-dimensional matrices were zero-filled twice in the t_1 and once in t_2 dimensions. For the first Fourier transform, data was multiplied by both trapezoidal and double-exponential window functions; for the second, only a trapezoidal multiplication was used. Chemical shifts were referenced to tetramethylsilane (TMS) using dioxan as an internal standard at 3.743 p.p.m.

RESULTS

Biological activity

As shown in Table 1, while αCGRP was found to stimulate adenylate cyclase in L6 myocytes (i.e. increase the cellular content of cyclic AMP), the desF-NH_2-αCGRP was unable to do this, above a background represented by that generated by the control peptide, BK. The latter peptide, which is unrelated to CGRP in sequence or structure, is thought to act in a number of different cell types through its own specific receptors and a different second-messenger system which involves phospholipase C and inositol phosphate turnover (Jackson et al., 1987; Sharif et al., 1988; Voyno-Yasenetskaya et al., 1989). The low-level effect of desF-NH_2-αCGRP on cyclic AMP concentration could not be titrated with a range of peptide concentrations, again indicating no effect due to the action of desF-NH_2-αCGRP on adenylate cyclase. Furthermore, desF-NH_2-αCGRP did not significantly antagonize the stimulatory effects of αCGRP on adenylate cyclase, even when in 100-fold molar excess over the latter. In summary the peptide desF-NH_2-αCGRP showed no significant interaction with the CGRP receptors on L6 myocytes. This conclusion is in agreement with results of Poyner et al. (1992).

C.d. studies

The far-u.v. c.d. spectra of CGRP and desF-NH_2-CGRP at 20 °C are shown in Figure 1. Parts (a), (b) and (c) of this Figure refer to spectra obtained in buffer (20 mM sodium phosphate, pH 7.0), buffer containing 50% (v/v) TFE or buffer containing 0.4% (w/v) SDS respectively. The results of applying the CONTIN procedure (Provencher and Glöckner, 1981) to the spectra obtained at 20 °C and 37 °C (the temperature at which the biological activity was studied) are shown in Table 2.

It should be noted that, in each case when the temperature was lowered from 37 °C back to 20 °C, the spectrum was superimposable with that previously recorded earlier at the lower temperature, indicating that any small changes in structure with temperature were completely reversible.

The helix content reported here for αCGRP is somewhat lower than that reported earlier, the presently reported intensities of the spectra at the minima (200–210 nm under the various conditions) being some 15% lower than previously found. This is probably due to our present use of lower wavelengths (down to 190 nm), which provide improved estimates of secondary structure (as discussed by Johnson, 1988). Small differences in experimental conditions could also contribute to small differences in result.

Importantly, no consistent differences were observed between the secondary structures of αCGRP and desF-NH_2-αCGRP at 20 °C or at 37 °C, whether in buffer alone or in the presence of TFE or SDS. The presence of TFE allows estimation of the helix-forming potential of the peptides, and SDS provides an environment presumably resembling that of a membrane-bound receptor.

N.m.r. studies

The one-dimensional ^{1}H-n.m.r. spectrum obtained in water at 27 °C of desF-NH_2-αCGRP and αCGRP showed a number of chemical-shift differences. The changes in the amide and α-CH regions of the spectra appear small, but are difficult to resolve because of resonance overlap. More distinct changes are observed in the aromatic and methyl regions, where resonances from side chains occur. At least some of these shifts are likely to arise directly from changes in ring-current effects as a consequence of a removal of the N-terminal phenylalanine residue.

Similar differences also arise in the spectra of the two peptides obtained in 50% (v/v) TFE/water mixtures. In order to make a more detailed quantitative analysis of these results, two-dimensional n.m.r. spectra were recorded and analysed.

Figure 2 shows the fingerprint region of the preTOCSY COSY spectrum of desF-NH_2-αCGRP in 50% (v/v) TFE/water at pH 3.7. The COSY experiment enables connectivities between *J*-

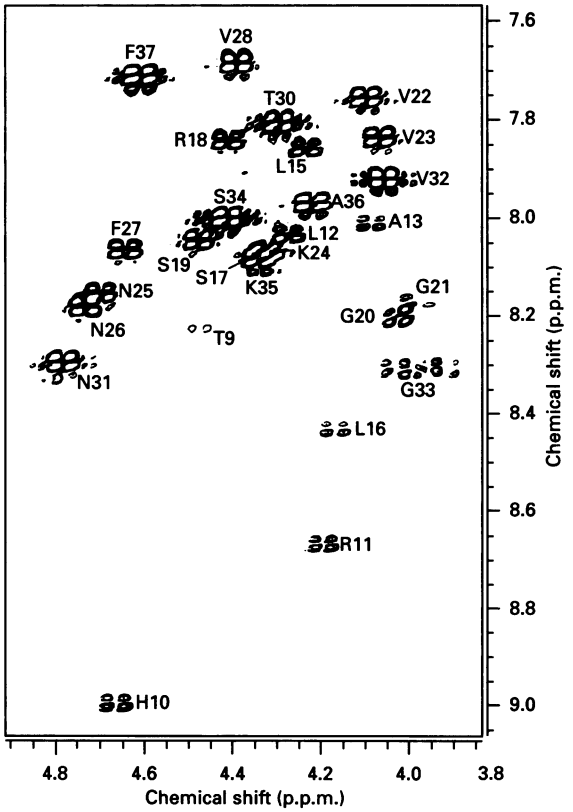

Figure 2 Fingerprint region of the 500 MHz preTOCSY COSY spectrum of desF-NH_2-αCGRP in 50% (v/v) TFE/water mixtures at 27 °C

Spectra were recorded as described in the Methods section. The single-letter code for the amino acid residues are used to indicate the assignment of the NH/αH cross-peaks.

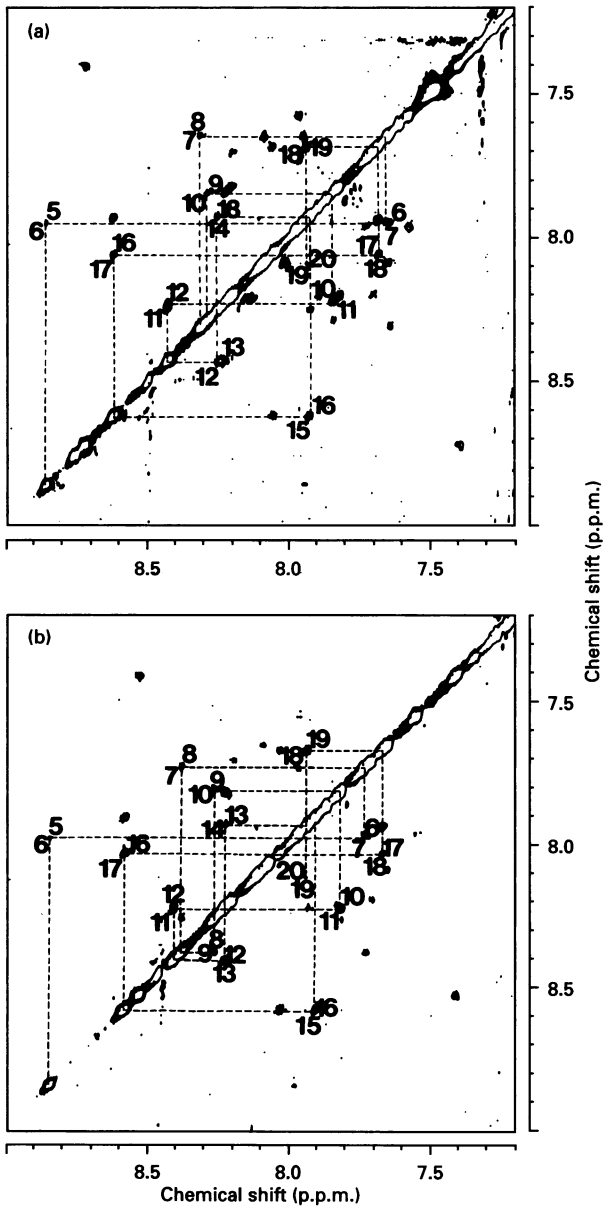

Figure 3 Amide region of the 500 MHz preTOCSY NOESY spectrum of desF-NH_2-αCGRP (a) and αCGRP (b) in 50% (v/v) TFE/water mixtures at 27 °C

The NOESY mixing time used was 200 ms. The cross-peaks are assigned with the residue numbers of the amino acids from whose amide protons they arise. The signal-to-noise ratios were 757 and 447 for the desF-NH_2-cCGRP and αCGRP spectra respectively. The signal-to-noise ratio was calculated using the UXNMR acquisition and processing package (Bruker). The value for noise was determined from the first $\frac{1}{16}$ of the spectrum which contained no resonance from the sample. The value for signal was determined from the whole spectrum, excluding the region from 5.0 to 4.6 p.p.m. (so as to exclude the solvent).

coupled protons to be established and hence allows assignment of spin systems (i.e. types of amino acids) to be made, but not sequence specific assignments. The fingerprint region of the COSY spectrum shows cross-peaks for each residue except the N-terminus and any proline residues; two cross-peaks are expected for each glycine due to the two α-protons. In desF-NH_2-αCGRP we therefore expect to see 40 cross-peaks in this region of the spectrum. Indeed, all expected cross-peaks were observed in desF-NH_2-αCGRP, although some were poorly resolved, owing to overlap (Figure 2).

Full sequence-specific assignments and comparison of the peptide structures were made by collection of two-dimensional nuclear-Overhauser-effect (n.O.e.) spectra (Figure 3), and the use of standard methods as described by Wuthrich (1986). A comparison of the spectra of αCGRP and desF-NH_2-αCGRP shows that only small chemical-shift changes occur at the C-terminus. These may be a direct effect of the loss of the phenylalanine residue. Small shifts were also seen in the vicinity of the N-terminus around Asp^3. These may be attributed to the fact that the experiments were carried out close to the pK_a of Asp^3, thus even small changes in pH will result in detectable shifts.

The n.O.e. experiment correlates protons which are close in space but not necessarily *J*-coupled. For example, in the fingerprint region we expect to see cross-peaks between amide and α-protons within the same residue and, in addition, cross-peaks from the α-proton of residue i to the amide of residue $i+1$. These sequential cross-peaks are strong for an extended conformation, but are weaker in helical-type secondary structure. In α-helices, strong sequential n.O.e.s are expected between amide protons on residues i to $i+1$. In addition, longer-range n.O.e.s from the α-proton of residue i to the amide proton of $i+3$ are often observed in an α-helix. A continuous stretch of amide-to-amide n.O.e.s is therefore diagnostic of helical secondary structure. Figure 3 displays the amide-to-amide region of the preTOCSY NOESY spectrum of desF-NH_2-αCGRP and αCGRP. The spectra are very similar in both the pattern and relative intensities of n.O.e.s. The few changes that are apparent result from chemical-shift changes around the loop close to Asp^3; for

example, the n.O.e. involving residues 8 and 9, which were obscured by the diagonal in αCGRP, can be clearly seen in desF-NH_2-αCGRP.

N.O.e.s characteristic of other types of secondary structure, and the N-terminal region loop are also observed (Figure 3). As found for αCGRP, there were no n.O.e.s between residues in different regions of the sequence for desF-NH_2-αCGRP that could be used to define a tertiary structure for the peptide (Breeze et al., 1991). A similar result has been obtained in the case of the related peptide antagonist CGRP 8–37 (J. A. M. Hubbard, C. M. Dobson and D. P. Raleigh, unpublished work). In the (Bu^t-Cys^{18}) 19–37 fragment of human βCGRP, a single n.O.e. between residues far removed in the linear sequence (between protons of Thr^{30} and Phe^{37}) was observed in a ROESY-type experiment by Sagoo et al. (1991). We observed no such interaction in our NOESY experiments, which we found to convey no less information than do ROESY experiments for these larger peptides. Indeed, if such an interaction was present in αCGRP, then the absence of Phe^{37} might give rise to a significant chemical-shift change in the methyl protons of Thr^{30}, due to loss of ring-current effects. That this is not the case suggests that a turn in this region of the peptide is not common in either αCGRP or desF-NH_2-αCGRP.

The N-terminal region of αCGRP exists in a relatively constrained conformation, as indicated by an examination of the $^3J_{NH\alpha}$ coupling constants (measured as apparent splittings in the one-dimensional spectrum) which were moderately large for Asn^3 and Thr^4 and small for Ala^5 (smaller than the linewidth of approx. 3 Hz).

In summary, by n.m.r., as by c.d., we observed no significant difference in secondary structure between αCGRP and desF-NH_2-αCGRP.

DISCUSSION

On the basis of data published to date, it is possible to divide the CGRP molecule into two functional domains. The first is the N-terminal seven residues, ACDTATC, in which residues 2 and 7 are disulphide-bonded. This intact S–S bond is important for biological activity on type 1 CGRP receptors at least (Dennis et al., 1989). The mechanism for this activity is obscure. It has been suggested that lutropin and follitropin may act on their respective receptors by virtue of a disulphide-bonded sequence of consensus CGXC(R or K) acting to isomerize disulphide groups in the receptor molecule, so causing structural changes and subsequent signal transduction (Boniface and Reichart, 1990). The disulphide in CGRP (or in the similar peptide amylin) does not seem to work in this way, however the peptide does not show any activity in an assay for protein disulphide-isomerase which involves renaturation of reduced and denatured ribonuclease (J. P. O'Connell, H. C. Hawkins and R. B. Freedman, unpublished work).

The remaining part of the molecule (8–37-NH_2) is not an agonist, but can bind to become an antagonist (Dennis et al., 1989; Donoso et al., 1990), albeit with lesser affinity than that of intact CGRP. The feature(s) of peptide 8–37-NH_2 which dictates its binding to the receptor is unclear. The α-helix in the region 8–18 may affect binding: sequence changes in this region have been found to alter biological activity (Lynch and Kaiser, 1988). It is conceivable that the α-helix content could affect the overall architecture of the molecule and hence its interaction with receptors, but it seems not to be the only feature which is important for binding to CGRP receptor(s). First, anti-CGRP antibodies, which can block CGRP's bioactivity, map functionally to the C-terminal ten-residue region (Andrew et al., 1990). Secondly, we show here that, when the C-terminal F-NH_2 is deleted, there is a large decrease in affinity for the CGRP receptor type 1, as present on L6 cells, and this is in agreement with similar observations made by Poyner et al. (1992). Our results suggest that the C-terminal F-NH_2 is a key feature which dictates the binding of CGRP to its specific receptor(s). There are two ways by which this might be the case: (i) by direct interactions of the F and/or NH_2 with groups on the receptor molecule; (ii) by the F and/or NH_2 determining secondary structure in the C-terminal region of the peptide which interacts with receptor.

Sagoo et al. (1991), in studying an N-terminally extended analogue of 19–37-NH_2 by n.m.r., found a suggestion of a loop or turn involving the C-terminal F-NH_2. Such a loop would be expected to be disrupted by removal of the F-NH_2, and this could explain a loss of ability to interact with receptor. We have studied the structure of whole CGRP and its desF-NH_2 analogue, by c.d. and n.m.r. and have no evidence to support the idea of the existence of a C-terminal loop structure.

The overall picture of αCGRP structure obtained is in agreement with previously reported data. Apart from the absence of signals directly attributable to the F-NH_2 itself, no definable difference was found between αCGRP and the des-F-NH_2 analogue. Therefore, unless it is the case that the deletion of the C-terminal F-NH_2 causes a change in CGRP structure which is too subtle for our current methods to detect, we would suggest that the F and/or NH_2 group itself interacts directly with CGRP receptor(s) and is an important determinant of binding of CGRP or antagonists derived from it.

The role of the C-terminal NH_2 group in CGRP has not previously been clear. That it protects the peptide from degradation by carboxypeptidases is a possibility, but that seems not to be its prime role. Our data suggest that its role is primarily one of interaction with CGRP receptor(s) and so contribution to the peptide's potency and specificity of action.

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