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The role of the disulphide bonds in endothelin-1

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Abstract—The smooth muscle contracting peptide endothelin-1 is characterized by the presence of two disulphide bonds and their importance for maintaining the agonist activity of the peptide was examined by synthesizing analogues of endothelin-1 lacking one or the other, or both, of these bonds. The circular dichroic spectra of these analogues (in which alanine residues replaced the appropriate cystines), [$Ala^{1,15}$]-, [$Ala^{3,11}$]- and [$Ala^{1,3,11,15}$]-endothelin-1 had features in common with that of endothelin-1. All three analogues exhibited at least some agonist activity in guinea-pig isolated trachea, but surprisingly endothelin-1 was a partial agonist in comparison with the analogue [$Ala^{3,11}$]endothelin-1. The disulphide bonds are therefore not absolutely essential for maintaining the tertiary structure necessary for agonist activity at endothelin-1 receptors in all tissues.

Endothelin is a bicyclic, 21 amino acid vasoconstrictor peptide isolated and characterized from culture medium porcine aortic endothelial cell by Yanagisawa et al (1988). Recently it has become apparent that endothelin is one of a family of peptides and the one originally described by Yanagisawa et al has been named endothelin-1 (Inoue et al 1989). Since endothelin-1 is presumably produced in the vicinity of vascular smooth muscle cells it may be involved in the control of vascular tone and, since it has been shown to either effect or have specific binding sites on numerous other cell types (Yanagisawa & Masaki 1989), may have multiple physiological and pathological roles. The primary structure of endothelin-1 is shown in Fig. 1. An unusual feature of this relatively short peptide is the presence of a disulphide bond between residues 1,15 and again between residues 3,11. While *multiple* disulphide bonds have been observed in certain small peptide venoms and toxins of marine or insect origin, they had not previously been found in mammalian peptides. Furthermore, the disulphide bonds of peptides such as somatostatin, oxytocin and atrial natriuretic factor for example are essential for maintaining their biologically active conformations (Veber & Saperstein 1979; Misono et al 1984). We were thus interested in examining the role of these disulphide bonds in maintaining the conformation of the molecule and their importance to the biological activity of the peptide. We report here the synthesis, conformation and biological activity of three endothelin analogues in which the disulphide bonds have been sequentially replaced with a pseudo-isoteric alanine residue.

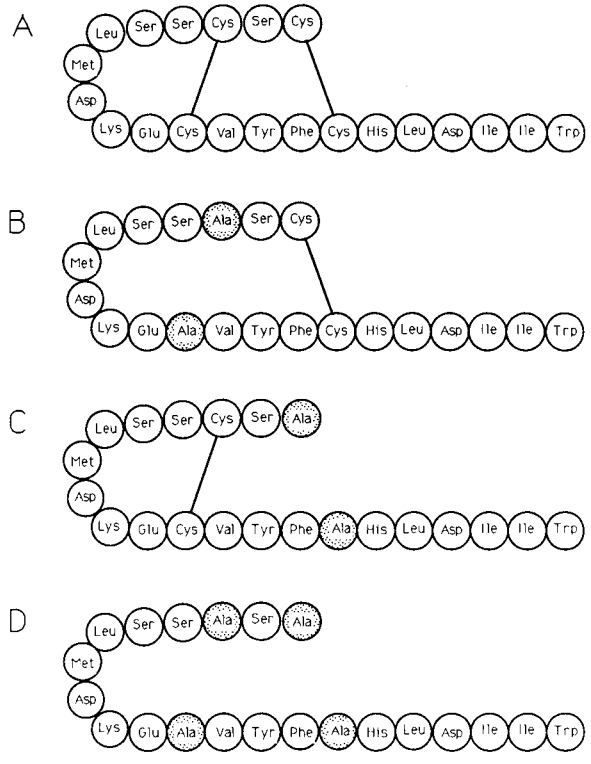

FIG. 1. A representation of the structures of (A) endothelin-1 and of the analogues (B) [$Ala^{3,11}$]endothelin-1, (C) [$Ala^{1,15}$] endothelin-1 and (D) [$Ala^{1,3,11,15}$]endothelin-1.

Materials and methods

Synthesis. Analogues of endothelin-1 were synthesized by standard solid phase synthesis techniques (Merrifield 1963) utilizing a t-butyloxycarbonyl/benzyl protection scheme on a 4-(oxymethyl)phenylacetamidomethyl (PAM) resin. Amino acids were double coupled using a two-fold excess of symmetrical anhydride and capped with acetylimidazole/1-hydroxybenzatriazole. The peptides were cleaved from the resin and globally deprotected by the "low-high" HF method (Tam et al 1983). Peptides were cyclized at high dilution with $K_3Fe(CN)_6$ at pH 8·5 and purified by a combination of Sephadex G25 gel filtration, SP Sephadex cation exchange chromatography and preparative HPLC on a C_{18} reverse phase column. Peptides were characterized by amino acid analysis, fast atom bombardment-mass spectroscopy (FAB-MS), TLC and HPLC.

Conformational studies. Circular dichroic (CD) spectra were measured on an AVIV Model 62DS spectropolarimeter from 300 nm to 180 nm in 0·5 nm intervals, at 24°C, 1·5 nm band width, 4 s averaging time using 1 mm circular cuvettes (Hellma). Samples of endothelin-1 or analogues (0·08 mg mL^{-1}) were dissolved in 10 mM Tris-HCl at pH 7·4 at 24°C or in distilled water. The CD spectra of buffer or water was subtracted from that of the sample after each scan. A total of 5 scans was averaged for each peptide and the data were fitted to a third order polynomial using the 'FIT' software supplied by AVIV. Predictions of secondary structure were calculated as described previously (Chou & Fasman 1978).

Guinea-pig trachea assay. Guinea-pigs, 250–300 g, were killed by a blow to the head and the trachea removed. Two zig-zag strips of trachea were prepared by the method of Mylechrane & Raper (1973) and the epithelium removed. The strips were suspended in organ baths containing physiological solution (composition in mM: NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1·2, $CaCl_2$ 1·25 and glucose 11·5) maintained at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2 under a tension of 0·5 g. Isotonic contractions to the cumulative additions of carbachol (10 nM–10 μM) were elicited after an initial 60 min equilibration

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period. Ninety min later when basal tone was stable, endothelin-1 or one of its analogues was added cumulatively to the bathing medium. Contractions were allowed to stabilize after each addition. In most experiments tissue was exposed only once to one peptide. EC50 values were calculated by interpolation from individual concentration-effect curves.

Endothelin-1 was purchased from Scientific Marketing, UK and carbachol from Sigma, France.

Results

Synthesis. Endothelin analogues were synthesized by standard solid phase synthesis techniques utilizing the PAM resin. The "low-high" HF cleavage method was used for global side-chain deprotection and cleavage of the peptide from the resin. In an early synthesis we observed that the standard hydrofluoric acid-anisole procedure at 0°C gave a by-product (43%) whose amino acid analysis, ultraviolet and FAB-MS spectra were consistent with a Friedel-Crafts acylation by anisole of the Glu^{10} side chain acylium ion (Feinberg & Merrifield 1974). No such by-products were obtained with the "low-high" hydrofluoric acid method. Peptides were cyclized at high dilution (50 μM) at pH 8·5 with $K_3Fe(CN)_6$ and purified by gel filtration, SP-Sephadex cation exchange chromatography and reverse phase HPLC on a Vydac C_{18} reverse phase column. Amino acid analysis after acid hydrolysis gave the expected molar ratios (±7·0%) of the constituent amino acids. Peptides were characterized by thin-layer chromatography, analytical RP-HPLC ($\lambda = 214$ nm), and FAB-MS.

Conformation studies. The circular dichroic spectra of endothelin-1 and analogues are presented in Fig. 2. The spectrum of the native hormone in either water or Tris-HCl buffer, pH 7·4 at 24°C is characterized by a negative absorption band at about 206 nm with a shoulder at about 223 nm and a weak positive band at 186 nm. [$Ala^{3,11}$]Endothelin-1 has qualitatively similar spectrum but is blue-shifted about 4 nm. Both [$Ala^{1,15}$] endothelin-1 and the tetra-alanine acyclic peptide are red shifted. Furthermore, the linear peptide has a large positive band at 193 nm. The Chou-Fasman calculations for endothelin-1 predict a β-turn involving residues Asp^8 to Cys^{11} ($< P > = 0{\cdot}93$) and a long β-sheet region extending from Val^{11} to the C-terminal tryptophan residue ($< P > = 1{\cdot}31$). Similar secondary structural features are predicted for the two cyclic analogues, while the linear peptide is calculated to have some α-helix character between residues Ser^5 and Glu^{10}, with β-sheet again extending from Ala^{11} to the C-terminus.

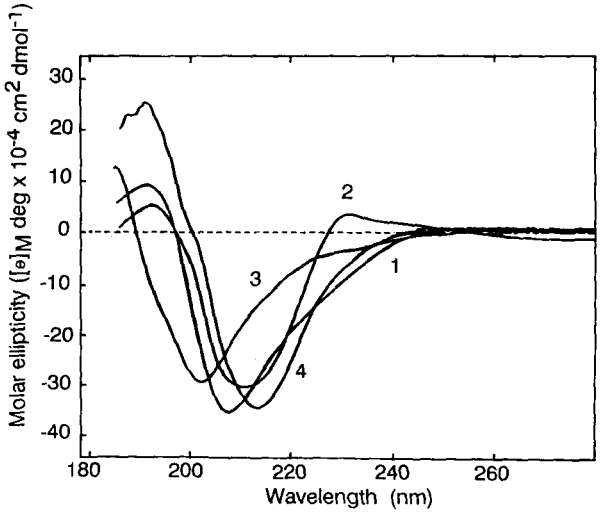

FIG. 2. Circular dichroic spectra of endothelin-1 (1), [$Ala^{1,15}$] endothelin-1 (2), [$Ala^{3,11}$]endothelin-1 (3) and [$Ala^{1,3,11,15}$]endothelin-1 (4). The spectra of endothelin-1 and [$Ala^{3,11}$]endothelin-1 are similar while those of [$Ala^{1,15}$]endothelin-1 and particularly of the acyclic analog [$Ala^{1,3,11,15}$]endothelin-1 show some marked differences compared to the native peptide.

Guinea-pig trachea assay. In the guinea-pig trachea endothelin-1 and its three analogues all elicited concentration-dependent increases in tension (Fig. 3). The EC50 value for endothelin-1 was 1·36 ± 0·31 nM. The maximal response elicited occurred with about 10 nM of the peptide and was smaller than that which could be elicited by a 100 fold greater concentration of [$Ala^{3,11}$]endothelin-1 (Fig. 3). That is, endothelin-1 appears to be a partial agonist in this tissue. Comparing equieffective concentrations of the peptides, their order of potency was endothelin-1 > [$Ala^{3,11}$]endothelin-1 > [$Ala^{1,15}$]endothelin-1 > [$Ala^{1,3,11,15}$]endothelin-1 and the potencies of the analogues relative to endothelin-1 (= 1) were about 0·13, 0·03 and 0·005, respectively (determined from responses equal to about 12% of the maximal response to carbachol; Fig. 3). Endothelin-1 was about 10 fold more potent than carbachol to elicit a similar response, but the maximal response elicited by the peptide was only about 30% of the carbachol maximum (Fig. 3). Maximal responses to the

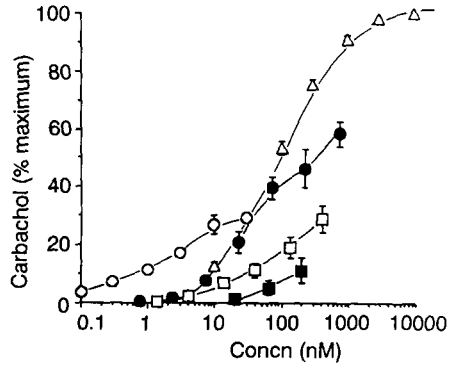

FIG. 3. Responses elicited by cumulative addition of carbachol (Δ), endothelin-1 (○), [$Ala^{3,11}$]endothelin-1 (●), [$Ala^{1,15}$]endothelin-1 (□) and [$Ala^{1,3,11,15}$]endothelin-1 (■) in guinea-pig tracheal preparations. Values are the means of at least 4 observations and vertical lines indicate s.e. mean where they exceed the size of the symbols.

analogues of endothelin-1 were not obtained with the highest concentrations used. In the presence of 3 nM endothelin-1, addition of concentrations of [$Ala^{3,11}$]endothelin-1 up to 0·75 μM elicited total contractions equal to 97 ± 7% of those elicited by 3 nM endothelin-1 alone. In two tissues precontracted by 0·2 μM [$Ala^{1,3,11,15}$]endothelin-1 (11% response), addition of 10 nM endothelin-1 elicited a further contraction, the total amounting to about 44% of the response induced by 10 μM carbachol.

Discussion

The recent isolation and characterization of the endothelin family of vasoconstrictor peptides (Yanagisawa et al 1988; Inoue et al 1989), which includes the sarafotoxins (Lee & Chiappinelli 1988; Takasaki et al 1988), represents a new addition to the growing list of known mammalian bioactive peptides. In this list there are many which contain a disulphide bond. However, the bicyclic nature of this relatively short 21 amino acid peptide is currently unique. The function(s) of the disulphide bonds in endothelin-1 also appears to be unusual. In previously known

cyclic peptides (somatostatin, oxytocin, atrial natriuretic factor, for example) the disulphide bond plays a crucial role in maintaining the biologically active conformation, with simple linear analogues having much reduced binding affinity and biological activity (see for example Veber & Saperstein 1979; Misono et al 1984).

In the guinea-pig trachea assay all three analogues were found to be agonists, although somewhat less potent than endothelin-1 itself. All three peptides are also agonists in the rat mesenteric and hindquarters vasculature, although [$Ala^{1,3,11,15}$]endothelin-1 behaves as a partial agonist (Randall et al 1989).

However, in these latter preparations, while [$Ala^{3,11}$]- and [$Ala^{1,15}$]endothelin-1 were respectively about 10 and 50 fold less potent than endothelin-1, [$Ala^{1,3,11,15}$]endothelin-1 was about equipotent with endothelin-1. The affinities of [$Ala^{3,11}$]- and [$Ala^{1,15}$]endothelin-1 for [^{125}I]endothelin-1 specific binding sites on rat cerebellar membranes are also respectively about 3 and 13 fold less than that of endothelin-1, and that of [$Ala^{1,3,11,15}$]endothelin-1 was about the same as endothelin-1 itself (Hiley et al 1990).

Thus, the removal of one or other of the disulphide bonds results in only modest reductions of the affinity and efficacy of the peptide. Surprisingly, removal of both disulphide bonds reduces efficacy in some instances (Randall et al 1989) with little or no effect on affinity in brain tissue (Hiley et al 1990). In the trachea, potency is evidently reduced, but not abolished, and affinity for [^{125}I]endothelin-1 binding sites is reduced (R. Jones, personal communication). Others have found that preventing formation of one or the other of the disulphide bonds with blocking groups on the cysteine residues results in complete loss of activity of the peptide (Hirata et al 1989), while reduction of both bonds resulted in about a 200 fold decrease in potency and reduced efficacy (Kimura et al 1988). This latter result is similar to that seen in trachea.

We have obtained the CD spectra of endothelin and analogues to examine the importance of the disulphide bonds in maintaining a defined conformation. The minimum in the negative absorption band is of approximately the same intensity for all peptides although it is blue- or red-shifted 3–10 nm relative to endothelin, depending on the analogue. The linear peptide also has a large positive absorption band at 193 nm. While structural predictions from CD data are difficult, the spectrum for the linear peptide is quite characteristic of β-sheet conformation (Woody 1985). Furthermore, of the four peptides, Chou-Fasman calculations predict the linear peptide to have the greatest portion as β-sheet structure (52%). The cyclic peptides are also predicted to contain β-sheet in the same region of the molecule, but the presence of the disulphide bond(s) at positions 3,11 or 1,15 or at both would likely alter the local conformation in this region and decrease the β-sheet contribution. The cyclic peptides are also predicted to contain a β-turn structure at residues 8–11. Although the CD spectra might be expected to reveal the presence of such a structure, the broad range of conformations that are described as β-turns give rise to CD patterns that differ in many ways.

Although the CD spectra of endothelin and mono-cyclic analogues are qualitatively similar, these peptides are short and small differences could represent substantial structural changes. Nevertheless, the similarities in the spectra suggest that the analogues might assume a solution conformation near to that of the native hormone, independent of some of the disulphide bonds. Such a structure could then account for the unexpected biological data indicating only partial loss in binding affinity and biological activity.

Another surprising observation was that endothelin-1 is apparently a partial agonist in tracheal smooth muscle. Maximal responses elicited by endothelin-1 were less than those that could be elicited by one of its analogues (Fig. 3) and it acted as an antagonist of [$Ala^{3,11}$]endothelin-1 as would be expected of a partial agonist (Bowman & Rand 1980). This might suggest that an endothelin other than endothelin-1 is the physiological agonist in this tissue.

Overall the results demonstrate that unlike other disulphide-containing peptides, endothelin-1 does not absolutely rely on these bonds to maintain a tertiary structure with affinity for specific binding sites or functional receptors.

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