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Binding of [^{125}I]-endothelin-1 to rat cerebellar homogenates and its interactions with some analogues

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1 [^{125}I]-endothelin-1, over the concentration range 6 pM–10 nM, bound to a single site in homogenates of rat cerebellum with high affinity ($K_d = 2.8 \pm 0.6 \times 10^{-10}$ M). The site was present in a concentration of 321 ± 58 fmol mg^{-1} protein.

2 The rates of association and dissociation of [^{125}I]-endothelin-1 with the binding site were slow (at 25°C, $k_{+1} = 8.0 \pm 1.3 \times 10^5$ M^{-1}s^{-1}; $k_{-1} = 2.6 \times 10^{-4}$ s^{-1}) and, on addition of a maximally displacing concentration of endothelin-1 (100 nM), 94.0 ± 8.4% of the [^{125}I]-endothelin-1 was still bound after 14 h.

3 [^{125}I]-endothelin-1 binding was inhibited by a number of naturally occurring or genetically encoded members of the endothelin/sarafotoxin family of peptides. The order of potency was endothelin-3 = sarafotoxin S6b > endothelin-2 = endothelin-1 ≫ porcine proendothelin$_{1-39}$.

4 Binding was also inhibited by analogues in which either one or both of the cystine disulphide bridges had been replaced by substitution with 2 or 4 alanine residues. The tetra-alanyl substituted analogue, [Ala^{1,3,11,15}]endothelin-1, was equipotent with endothelin-1 at inhibiting the binding of [^{125}I]-endothelin-1. [Ala^{3,11}]endothelin-1 and [Ala^{1,15}]endothelin-1, analogues which each contained one of the disulphide bridges from the parent peptide, were respectively 3 and 14 times less potent than the parent peptide. An analogue in which the Glu^{10} residue had been anisylated was 25 fold less potent than endothelin-1.

5 It is concluded that the structural requirements for binding to the cerebellar sites for [^{125}I]-endothelin-1 do not require the presence of the disulphide bridges characteristic of the endothelin/sarafotoxin family. Rather, the binding may be more sensitive to the presence of bulky side chain substituents, at least in the smaller intramolecular loop.

Introduction

Yanagisawa *et al.* (1988b) isolated a very potent pressor peptide, which they named endothelin, from medium conditioned by pig aortic endothelial cells. It has since been established that this is one of a group of peptides, the endothelin/sarafotoxin family, that occur either naturally or may be found encoded in mammalian genomic libraries. Each member of this group consists of 21 amino acids and has 2 disulphide bridges which are between residues 1 and 15 and between residues 3 and 11. The three different peptides encoded in the human genome have been denoted endothelin-1, endothelin-2 and endothelin-3 (Inoue *et al.*, 1989). Of these peptides, coding for a sequence identical to human endothelin-1 has also been found in the pig (Yanagisawa *et al.*, 1988b), for endothelin-1 and endothelin-2 in the dog (Itoh *et al.*, 1989; Kimura *et al.*, 1989) and for endothelin-3 in the rat and rabbit (Yanagisawa *et al.*, 1988a; Ohkubo *et al.*, 1990). The sequence for a fourth mammalian peptide, vasoactive intestinal contractor, has been detected in the mouse genome (Saida *et al.*, 1989). In addition to these mammalian peptides, there exist closely homologous peptides, the sarafotoxins S6, which are found in the venom of the burrowing asp, *Atractaspis engaddensis* (Kloog *et al.*, 1988).

In view of the conservation of the disulphide bridges between the members of the endothelin/sarafotoxin family, interest has centred on their importance for biological activity. Kimura *et al.* (1988) found that peptides in which the cysteines which form the disulphide bridges had been left blocked by carboxamidomethyl groups were at least 1000 fold less active as vasoconstrictor agents on pig coronary artery. However, the blocking groups are very bulky and may interfere with the structure of the peptides in ways other than those simply associated with the loss of the disulphide bridges. Accordingly, a series of analogues of human endothelin-1 have been synthesized in which the cystine residues have been replaced by pairs of alanines. Those analogues which contain only one of the disulphide bridges have been found to be only 10 to 40 fold less active than endothelin-1 itself not only in the rat aorta (Topouzis *et al.*, 1989) and rat mesenteric arterial bed (Hiley *et al.*, 1989a; Randall *et al.*, 1989) but also in preparations such as guinea-pig trachea (Pelton & Miller, unpublished observations) and the field stimulated guinea-pig ileum and rat vas deferens (Hiley *et al.*, 1989b).

The discovery that endothelin and its analogues could act on nerve/muscle preparations was particularly interesting in view of the observation that binding sites for [^{125}I]-endothelin-1 have been identified in many tissues, including brain and kidney, with distributions that do not suggest that they are exclusively associated with the blood vessels of those tissues (Davenport *et al.*, 1989a,b; Jones *et al.*, 1989a,b). In the rat brain, the greatest concentration of [^{125}I]-endothelin-1 binding sites was found in the hind brain, particularly in the cerebellum (Jones *et al.*, 1989b). It was therefore of interest to study the interactions between [^{125}I]-endothelin-1, its naturally encoded relations and its alanyl-substituted analogues in rat brain.

A preliminary communication of this work was made to the December 1988 Meeting of the British Pharmacological Society (Jones *et al.*, 1989c).

Methods

Membrane preparation

Male Sprague-Dawley rats (185–250 g; Charles Rivers France) were killed by stunning and decapitation. The cerebellum was then removed and homogenized in 10 vol. ice-cold 50 mM HEPES ({N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]}; Sigma Chimie, La Verpillière, France) buffer (pH 7.4) containing 1 mM 1,10 phenanthroline (Sigma). The

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homogenates were centrifuged at 1000 g for 10 min at 4°C and the binding studies were carried out on the resulting supernatant. Protein content was determined by the method of Lowry et al. (1951).

[^{125}I]-endothelin-1

Human endothelin-1 (1 nmol; Peptide Institute, Osaka, Japan) was iodinated by the method of McKee et al. (1986) using a 10 s reaction time with chloramine-T (Aldrich Chemical Co., Strasbourg, France) and sodium [^{125}I]-iodide (481–574 MBq μg^{-1}; Amersham, Les Ulis, France). The reaction was stopped by the addition of sodium metabisulphite. Bovine serum albumin (500 mg in 500 μl 50 mM Tris buffer, pH 7.6) was added to the reaction mixture as a carrier and the reaction mixture was then passed down a Sephadex G-15 column. Elution was with the Tris buffer and the product was then subjected to reverse phase high performance liquid chromatography using a Vydac (Hesperia, CA, U.S.A.) Type 218TP54 C-18 reverse phase column for protein and peptide separation; the mobile phase consisted of a linear gradient passing from 75% to 50% H_2O in CH_3CN (v/v) containing 0.1% trifluoroacetic acid (v/v) and the flow rate was 2 ml min^{-1}.

In later experiments, [^{125}I]-endothelin-1 was purchased from Amersham (specific activity 74 TBq $mmol^{-1}$) and no differences were found between the performance of this radioligand and that prepared in the laboratory.

Synthesis of analogues of endothelin-1

Analogues of endothelin-1 were synthesized by standard solid-phase synthetic techniques using an automated peptide synthesizer employing Boc/Bzl chemistry (Merrifield, 1963; Stewart & Young, 1984) and t-butyloxycarbonyltryptophyl-4-(oxymethyl)phenylacetamidomethyl resin. The peptides were deprotected and removed from the resin with the two-step 'low-high' HF method (Tam et al., 1983). After cyclization with 0.01 M $K_3Fe(CN)_6$ at pH 8.5 (Pelton et al., 1986), the analogues were purified by gel filtration and preparative reverse-phase high pressure liquid chromatography. Amino acid analysis after acid hydrolysis gave the proper molar ratios (± 8.0%) of the constituent amino acids. Molecular ions and fragmentation patterns determined by fast atom bombardment-mass spectroscopy were consistent with the amino acid composition and sequence.

Binding studies

Incubations were carried out in triplicate at 25°C in a volume of 0.5 ml in microcentrifuge tubes using 150–700 μg protein. For inhibition experiments with unlabelled ligands membranes were incubated with 20–50 pM [^{125}I]-endothelin-1; under these conditions the radiolabel occupies about 3–6% of the binding sites in the absence of a competing ligand. Non-specific binding was defined with 0.1 μM unlabelled endothelin-1 and the reaction was started in all cases by the addition of the cerebellar homogenate. An incubation time of 2 h was used for inhibition studies with unlabelled peptides and for saturation experiments. Other times of incubation were used as appropriate for kinetic experiments. The reaction was terminated by centrifugation in a Beckman Microfuge B for 2 min at 14000 g; the pellets were washed 3 times by dipping the microcentrifuge tubes into ice-cold incubation medium (50 mM HEPES, pH 7.4; 0.1% bovine serum albumin; 1 mM 1,10 phenanthroline; 140 $\mu g\,ml^{-1}$ bacitracin: all from Sigma) and then the residual radioactivity in the pellets was determined by counting in a Packard Autogamma 5000 γ-scintillation spectrometer.

Kinetic experiments were carried out by determining the amount of specific binding over several time points with 6 different concentrations of [^{125}I]-endothelin-1 ranging from 18–450 pM. The rate constant for the formation of the [^{125}I]-endothelin-1/binding site complex (k_{obs}) was calculated by use of LIGAND (see below) and the forward and backward rate constants (k_{+1} and k_{-1}) were determined by linear regression of k_{obs} against concentration of radioligand.

Analysis of data

Kinetic data were analysed by non-linear least squares fitting with the KINETIC program (McPherson, 1985) running on a Compaq 386 PC AT compatible computer. Saturation data were initially analysed with EBDA (McPherson, 1985) and then subjected to non-linear least squares curve fitting by use of LIGAND (Munson & Rodbard, 1980).

Inhibition data were also subjected to preliminary analysis with EBDA and then the resulting inhibition data were fitted to a logistic equation:

$$\%\ \text{inhibition of specific binding} = \frac{100}{\{1 - (IC_{50}/X)^{n_H}\}}$$

where IC_{50} is the concentration of the unlabelled ligand causing 50% inhibition of the binding of the radioligand, X is the concentration of the unlabelled ligand and n_H is the Hill slope of the curve. Non-linear curve-fitting was carried out with the RS/1 data analysis package (BBN Software Products Corporation, Cambridge, MA, U.S.A.). The K_i was calculated from the resulting IC_{50} by use of the procedure of Munson & Rodbard (1988).

Results

Rate of binding of [^{125}I]-endothelin-1 to cerebellar binding sites

The time course of labelling of binding sites by 30 pM [^{125}I]-endothelin-1 in rat cerebellar homogenates was very slow. From a series of experiments using 6 different concentrations of the radioligand, the forward rate constant was calculated to be $8.0 \pm 1.3 \times 10^5\ M^{-1}\,s^{-1}$ and the backward rate constant to be $2.6 \pm 0.3 \times 10^{-4}\,s^{-1}$; this gives an estimate for the binding constant of 3.2×10^{-10} M. In dissociation experiments, 100 nM endothelin-1 was added to the membrane/[^{125}I]-endothelin-1 reaction mix after the 2 h association phase, dissociation of the bound [^{125}I]-endothothelin-1 was very slow with 94.0 ± 8.4% (n = 3) remaining bound to the membranes after 14 h.

Saturation analysis of [^{125}I]-endothelin-1 binding to cerebellar sites

Figure 1 shows the saturation curve and the Scatchard analysis for an experiment in which cerebellar membranes were incubated with 6 pM–10 nM [^{125}I]-endothelin-1. The binding appears to be a single class of site and in 5 experiments the equilibrium dissociation constant was 2.8 $\pm 0.6 \times 10^{-10}$ M, the maximal specific binding was 321 ± 58 fmol mg^{-1} protein and the Hill slope was 0.92 ± 0.04. Figure 1 shows the results from one of two saturation experiments in which 1 nM sarafotoxin S6b was present and it can be seen that, in its presence, the Scatchard plot for [^{125}I]-endothelin-1 is linear and the intercept on the abscissa scale is very close to that for the iodinated ligand alone. Analysis of these two competition experiments gave values of the dissociation constant for sarafotoxin S6b of 3.2 and 1.9×10^{-10} M and the Hill slopes for the binding of [^{125}]-endothelin-1 were 0.92 and 1.04. The maximal specific binding of [^{125}I]-endothelin-1 in the presence of sarafotoxin S6b in these two experiments was 510 and 394 fmol mg^{-1} protein as compared to the values of 473 and 340 fmol mg^{-1} protein found with [^{125}I]-endothelin-1 alone in the same two homogenates.

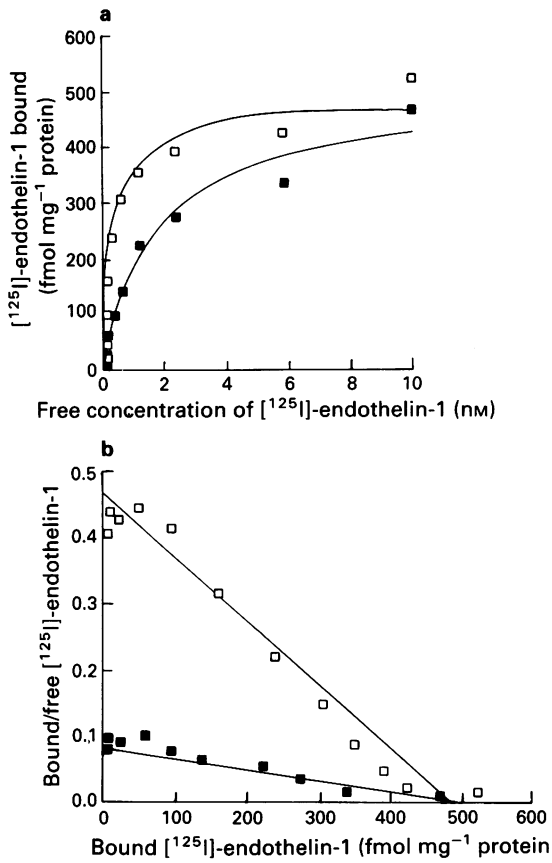

Figure 1 Results from a single experiment in which cerebellar membranes were incubated with 6 pM–10 nM [^{125}I]-endothelin-1 in the absence (□) and presence (■) of 1 nM sarafotoxin S6b. (a) Saturation curves with the lines showing the curves obtained from the fitting procedure. (b) Scatchard plots of the same data showing the linear least squares regression lines. The points are the mean of triplicate determinations.

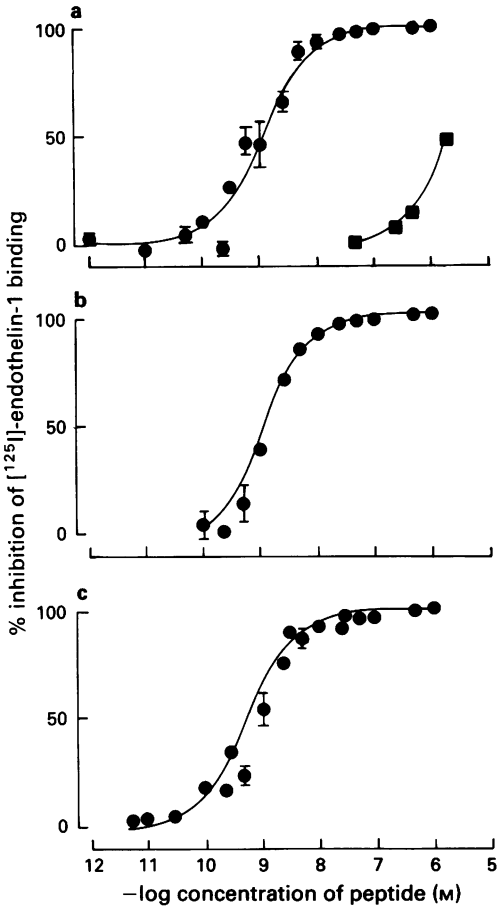

Figure 2 Inhibition of specific [^{125}I]-endothelin-1 binding to homogenates of rat cerebellum by members of the endothelin family. (a) Inhibition by endothelin-1 (●; $n = 6$) and porcine proendothelin$_{1-39}$ (■; $n = 4$); (b) inhibition by endothelin-2 ($n = 3$): (c) inhibition by endothelin-3 ($n = 6$). The points are the mean of n determinations on separate homogenates and the bars show s.e.mean; where no bar is shown the s.e.mean lies within the size of the symbol. The lines show the curves of best fit obtained from the fitting procedure.

Displacement of [^{125}I]-endothelin-1 by unlabelled analogues

Table 1 shows that [^{125}I]-endothelin-1 binding was inhibited by all the analogues used in the study; all of the peptides inhibited the binding of the radioligand to the same extent. The most potent inhibitors of its binding were the snake cardiotoxin sarafotoxin S6b and endothelin-3 with endothelin-1 itself and endothelin-2 having dissociation constants that were 3 to 4 fold greater (Figure 2). It is particularly interesting to note that all three of the alanyl-substituted analogues of endothelin-1 were able to inhibit specific [^{125}I]-endothelin-1 binding to sites in cerebellar homogenates (Figure 3). Thus, [Ala^{3,11}]endothelin-1 was about 3 fold less potent than the parent peptide and [Ala^{1,15}]endothelin-1 had an equilibrium dissociation constant 14 times greater than that of endothelin-1. However, the most interesting observation is that the ana-

Table 1 Binding constants and Hill slopes for inhibition of [^{125}I]-endothelin-1 by members of the endothelin/sarafotoxin family and by alanyl-substituted analogues of endothelin-1

	K_i (nM)	*Hill slope*	n
Endothelin-1	0.83 ± 0.15	1.1 ± 0.1	6
Endothelin-2	1.02 ± 0.16	1.0 ± 0.1	3
Endothelin-3	0.24 ± 0.05	0.9 ± 0.1	6
Sarafotoxin S6b	0.23 ± 0.06	1.1 ± 0.1	6
Porcine proendothelin$_{1-39}$	1250 ± 210	1.1 ± 0.2	4
[Ala^{1,15}]endothelin-1	11.5 ± 2.7	1.5 ± 0.1**	7
[Ala^{3,11}]endothelin-1	2.98 ± 0.3	1.3 ± 0.1**	8
[Ala^{1,3,11,15}]endothelin-1	0.72 ± 0.11	0.9 ± 0.1	7
[Acetamidomethyl-Cys^{3,11}]endothelin-1	8.1 ± 0.8	0.8 ± 0.1*	6
[Anisyl-Glu^{10}]endothelin-1	20.7 ± 2.1	0.8 ± 0.1*	3

K_i is the dissociation constant for the inhibition of [^{125}I]-endothelin-1 binding by the unlabelled peptide. Values are given as the mean ± s.e.mean and n represents the number of homogenates used. The values were determined by non-linear least squares curve fitting to a logistic equation.

Significant differences from 1 in Hill slopes are indicated by: * $P < 0.05$; ** $P < 0.01$. These were tested by carrying out curve-fitting with the Hill slope constrained to 1 and determining if the sum of squares of residuals was significantly greater than when the Hill slope was allowed to vary.

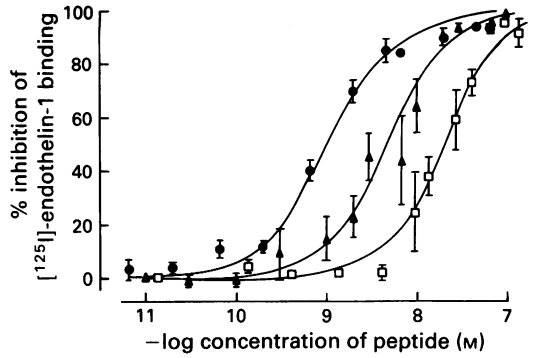

Figure 3 Inhibition of [^{125}I]-endothelin-1 binding to homogenates of rat cerebellum by three alanyl-substituted analogues of endothelin-1. (●) [Ala^{1,3,11,15}]endothelin-1 ($n = 7$); (▲) [Ala^{3,11}]endothelin-1 ($n = 8$); (□) [Ala^{1,15}]endothelin-1 ($n = 7$). The lines are the curves of best fit obtained from the fitting procedure. The points are the mean• of n determinations on separate homogenates and the bars show s.e.mean; where no bar is shown the s.e.mean lies within the size of the symbol.

logue which is devoid of disulphide bridges, [Ala^{1,3,11,15}]-endothelin-1, was equipotent with endothelin-1.

Comparison of [Ala^{3,11}]endothelin-1 with an analogue of endothelin-1 in which acetamidomethyl blocking groups were present on cysteine residues 3 and 11 shows that these bulky groups further reduced the inhibitory potency of the peptide by a factor of 2.7. Another analogue modified in the region of the disulphide bridge forming the smaller intramolecular loop, [anisyl-Glu^{10}]endothelin-1, was the least potent of the peptides studied being 25 fold less potent than endothelin-1.

The Hill slopes of the inhibition curves were not significantly different from 1 for endothelin-1, endothelin-2, endothelin-3, sarafotoxin S6b or the tetra-alanyl analogue of endothelin-1. The two dialanyl-substituted analogues of endothelin-1 had slopes which were significantly greater than unity whilst those of [acetamidomethyl-Cys^{3,11}]endothelin-1 and [anisyl-Glu^{10}]endothelin-1 were significantly less than 1.

Discussion

The rat cerebellum has previously been shown by autoradiography to contain high concentrations of binding sites for [^{125}I]-endothelin-1 (Davenport *et al.*, 1989b; Hoyer *et al.*, 1989; Jones *et al.*, 1989b). The binding site in rat cerebellar slices is of high affinity and the radioligand does not dissociate readily from the binding site. The present study shows that homogenates of rat cerebellum also contain a binding site for [^{125}I]-endothelin-1 which is of high affinity. Over the range 25 pM–10 nM the binding is to a single site and the affinity of this site, approximately 0.4 nM, is similar regardless of whether it is determined by saturation or by analysis of the kinetics of onset.

The interaction between the radioligand and its binding site can be inhibited by a number of peptide analogues of endothelin-1. The snake cardiotoxin, sarafotoxin S6b, and endothelin-3 were the most potent inhibitors of [^{125}I]-endothelin-1 binding, both being more potent than endothelin-1 itself. Endothelin-2, the third peptide encoded in the human genome (Inoue *et al.*, 1989), was approximately equipotent with the parent peptide; thus all four of these naturally occurring or genetically encoded peptides have approximately equal affinity at this binding site.

Mutual inhibition of endothelin-1 and sarafotoxin S6b specific binding in rat brain has also been demonstrated by Ambar *et al.* (1989), but they found that endothelin-1 was more potent than the toxin at inhibiting [^{125}I]-endothelin-1 binding in whole brain homogenates and they showed that the two peptides were somewhat less potent than was found in the present study. In other tissues, such as rat heart, sarafotoxin S6b and endothelin-1 have been found to be approximately equipotent at inhibiting [^{125}I]-endothelin-1 specific binding (Gu *et al.*, 1989). In the rat cerebellar homogenates, the presence of 1 nM sarafotoxin S6b in saturation experiments did not alter either the maximal binding or Hill slope for [^{125}I]-endothelin-1, which confirms that there was simple competition for a single site.

It has been suggested that the peptides may be produced from a 'pre-pro' form and the sequences for the pig and human forms of the putative precursors of endothelin-1 have been determined (Yanagisawa *et al.*, 1988b; Itoh *et al.*, 1988). Pig proendothelin-1 is a 39 amino acid peptide and it was found to be very weak at inhibiting the binding of [^{125}I]-endothelin-1, having an affinity 1000 fold less than that of endothelin-1 and the other 21 amino acid peptides. However, the putative human precursor, proendothelin-1_{1-38}, has vasoconstrictor actions in the rat isolated mesenteric bed (Douglas & Hiley, 1990), where it is at least 300 times less potent than endothelin-1, and in the rat aorta in which it is 100 fold less potent than the parent peptide (Kashiwabara *et al.*, 1989). Thus there may be differences in potency between the two proendothelins.

In view of the fact that the endothelin/sarafotoxin family of peptides are characterized by the possession of two disulphide bridges, between positions 1 and 15, and 3 and 11, it was of particular interest to determine the activities of peptide analogues of endothelin-1 which lack one or both of these bridges. Four of these peptides were investigated. One, [acetamidomethyl-Cys^{3,11}]endothelin-1, had the same amino acid sequence as the parent peptide but the formation of the 3-11 disulphide bridge was prevented by the use of acetamidomethyl blocking groups. In the other 3 compounds cysteine residues were replaced with alanine, which is pseudoisoteric with cysteine, in order to minimize the disruption to the secondary structure. All three of these compounds were able to inhibit the binding of [^{125}I]-endothelin-1 to its binding sites in cerebellum and they inhibited the radiolabelling to the same extent as did endothelin-1 itself and the other 21 amino acid peptides encoded in the human genome. [Ala^{3,11}]endothelin-1, which possesses a single disulphide bridge between positions 1 and 15, was approximately 3 fold less potent than endothelin-1 itself but was in turn 3 times more active than the compound in which the formation of the 3-11 bridge had been prevented by blocking groups. This suggests that the acetamidomethyl groups cause more disruption of the structure than can be attributed simply to the absence of the disulphide bond.

Indeed, [acetamidomethyl-Cys^{3,11}]endothelin-1 did not inhibit the binding of [^{125}I]-endothelin-1 to rat cultured vascular smooth cells in concentrations up to 0.1 μM, nor did it stimulate increases in intracellular calcium (Hirata *et al.*, 1989). Further indication of the sensitivity of the binding site to substitution in the smaller intramolecular loop near to position 11 came from the markedly lower potency at inhibiting the binding of the radiolabel found for [anisyl-Glu^{10}] endothelin-1. Thus it would seem that bulkiness of the amino acid residues in this region is of more consequence for binding potency than the 3-11 disulphide bridge itself. Another point of interest is that [anisyl-Glu^{10}]endothelin-1 and [acetamidomethyl-Cys^{3,11}]endothelin-1 were the only two of the agents investigated which had Hill slopes of interaction with [^{125}I]-endothelin-1 which were significantly less than unity; there is therefore the possibility that they do not interact with a single class of binding sites even though no heterogeneity of binding was observed with [^{125}I]-endothelin-1 itself.

Confirmation of the relative lack of importance of the disulphide bridges in determining binding potency came from the second peptide containing a single disulphide bridge due to alanine substitution; [Ala^{1,15}]endothelin-1 was 4 fold less potent than [Ala^{3,11}]endothelin-1 in inhibiting radioligand

binding. Thus, possession of a single disulphide bridge in itself does not greatly reduce the activity of this series of peptides at the cerebellar binding site. Further, perhaps the most surprising result was that the peptide devoid of disulphide bridges, [Ala^{1,3,11,15}]endothelin-1, was more potent than either of the single bridge compounds and was approximately equipotent with endothelin-1.

All the alanyl-substituted endothelin-1 analogues have been found to have biological activity. In rat aorta (Topouzis *et al.*, 1989), guinea-pig trachea (Pelton & Miller, unpublished observations) and rat perfused mesenteric arterial beds (Randall *et al.*, 1989) the order of potency at causing constriction is endothelin-1 > [Ala^{3,11}]endothelin-1 > [Ala^{1,15}]-endothelin-1. [Ala^{1,3,11,15}] endothelin-1 did not cause any constriction of the isolated Krebs-Henseleit perfused mesenteric bed, but it had agonist activity in guinea-pig trachea and acted as a partial agonist in the *in situ* blood perfused mesenteric bed where it had an ED_{50} equal to that of endothelin-1. It also had a pressor effect in the pithed rat in which it had one-fifth of the potency of the parent peptide (MacLean & Hiley, 1989). Thus the finding that it is equipotent at inhibiting [^{125}I]-endothelin-1 binding is consistent with this biological activity.

The results of this present study show that there is apparently a single class of binding sites for [^{125}I]-endothelin-1 in the rat cerebellum and that the radiolabel may be inhibited from binding to these sites by a number of peptide analogues. The structure-activity relationship for this displacement shows that the possession of disulphide bridges is not a strict requirement for binding to the site and this is consistent with observed biological activity in a number of tissues. Binding may, however, be sensitive to the possession of bulky substituents in the region of residues 3, 10 and 11.

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