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THE IMPORTANCE OF THE N-TERMINAL END OF ANGIOTENSIN II FOR ITS PRESSOR AND OXYTOCIC ACTIVITIES*

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Abstract—The importance of the N-terminal end of angiotensin II for its biological activity has been investigated by a comparative study of the pressor and oxytocic potencies of Val[5] angiotensin II and the following peptide analogues: (1) Asp(NH_2). Arg.Val.Tyr.Val.His.Pro.Phe; (2) Gly.Arg.Val.Tyr.Val.His.Pro.Phe; (3) Arg. Val.Tyr.Val.His.Pro.Phe; (4) Val.Tyr.Val.His.Pro.Phe. It was found that: (1) the absence of the N-terminal aspartyl residue of angiotensin II resulted in a parallel reduction of the pressor and oxytocic activities to 26 and 23 per cent, respectively; (2) the removal of the N-terminal aspartyl-arginyl portion of angiotensin II abolished the two activities; (3) the blocking of the free γ-carboxyl group of the aspartyl residue by an amide bond caused a greater reduction of oxytocic (73 per cent) than pressor activity (47 per cent).

IN A previous paper[1] we have reported the marked oxytocic activity of synthetic angiotensin II-amide[2] and studied the effect of enzymic and partial acid hydrolysis on the biological activities of the peptide. We have concluded, from such a study, that the C-terminal phenylalanine residue and the N-terminal aspartyl-arginyl moiety of angiotensin II are equally essential for both its pressor and oxytocic activities, but there was reason to believe that the N-terminal aspartyl residue contributed to the two activities to different extents.

In the present communication we report a further study of the relation between the N-terminal end of angiotensin II and its pressor and oxytocic activities, consisting of a comparison of the two activities in the five following peptides:‡ (1) Asp.Arg.Val. Tyr.Val.His.Pro.Phe (angiotensin); (2) Asp(NH_2).Arg.Val.Tyr.Val.His.Pro.Phe (angiotensinamide); (3) Gly.Arg.Val.Tyr.Val.His.Pro.Phe (glycyl octapeptide); (4) Arg.Val.Tyr.Val.His.Pro.Phe (arginyl heptapeptide); (5) Val.Tyr.Val.His. Pro.Phe (valyl hexapeptide).

EXPERIMENTAL

Materials

Val[5] angiotensin II (angiotensin), Val[5] angiotensin II-amide (angiotensinamide) and the octapeptide Gly.Arg.Val.Tyr.Val.His.Pro.Phe (glycyl octapeptide) were synthetic products kindly supplied by Dr. R. Schwyzer, Ciba Limited, Basel. These substances had been purified by counter-current distribution as free peptides, in the

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‡ The peptides are represented with the aid of the customary abbreviations for the amino acid residues,[3] with the N-terminal residues at the left of the sequences as written.

final stage of the synthesis, and contained from one to three molecules of water and from one to two molecules of acetic acid per molecule of peptide; their purity, checked by paper chromatography, was found to be greater than 99 per cent and, in the case of angiotensinamide, contamination by angiotensin was found to be less than 1 per cent.[4] Twice-crystallized trypsin, lot PM629, was acquired from the Worthington Biochemical Corporation, Freehold, New Jersey.

Methods

Trypsin digestion. One milligram of angiotensinamide was dissolved in 1·0 ml of a 0·05 M ammonium acetate–ammonium bicarbonate buffer of pH 7·7, and to this solution were added 0·05 ml of a trypsin solution containing 5 mg of protein per ml. The molar enzyme : substrate ratio was 1 : 90. After 6 hr at room temperature the reaction was stopped by the addition of 0·5 ml of glacial acetic acid and boiling for 5 min. The mixture was thoroughly evaporated in a desiccator and submitted to paper chromatography for the isolation of the valyl hexapeptide.

The phenylisothiocyanate method of Edman[5] for the stepwise degradation of peptides was modified as follows: 1 mg of angiotensinamide was dissolved in 10 ml of a buffer solution of pH 9·7 prepared by adding 0·7 ml of triethylamine to 2·5 ml of 2N acetic acid and diluting with water to 25 ml. One millilitre of a 20% (v/v) solution of phenylisothiocyanate in acetone was added to the angiotensinamide solution and the mixture shaken vigorously for 3 hr at 40 °C. One millilitre of water was added and the mixture was extracted with seven 5-ml portions of redistilled benzene. The aqueous phase was taken to dryness in a desiccator and redissolved in 5 ml of 3N HCl prepared from glass-distilled constant-boiling HCl. An aliquot of this solution was suitably diluted with 3N HCl and its absorption spectrum in the range 230–280 mμ was immediately determined with a Beckman model DU spectrophotometer. The change in the spectrum was followed by making readings at 30-min intervals, and the release of the phenylthiohydantoin was considered completed when a maximum at 265 mμ and a minimum at 245 mμ were attained. The acid solution was extracted with three 10-ml portions of redistilled ethyl acetate and the aqueous phase was taken to dryness in a rotary evaporator. The dry residue was submitted first to paper ionophoresis, then to paper chromatography for the isolation of the arginyl heptapeptide.

Paper ionophoresis[6] was done on Whatman 3MM paper with pyridine acetate buffer of pH 6·4 and a potential gradient of 15 V/cm for 3–4 hr. After the paper had dried, a guiding strip cut from each edge was sprayed with 0·1% alcoholic ninhydrin solution for the localization of the peptides, which were then eluted with water,[7] dried in a desiccator and redissolved in a small volume of water.

Preparative paper chromatography was done on Whatman 3MM filter paper with the solvent mixture butanol-acetic acid-water (63 : 10 : 27, v/v/v). The peptides were localized with ninhydrin on two guiding strips cut from each edge of the chromatogram, and eluted with water. The eluates were evaporated in a desiccator and redissolved in water. Aliquots of these solutions were taken for aminoacid analyses and for determination of biological activity.

Amino acid composition was determined by dissolving the peptide in a small amount of azeotropic HCl (6N); this solution was enclosed, under vacuum, in a vial and heated at 110 °C for 20 hr. The hydrolysate was evaporated to dryness and

chromatographed on Whatman no. 1 filter paper with butanol–acetic acid–water (63 : 10 : 27) simultaneously with several serial dilutions of a standard mixture containing equimolar amounts of the seven amino acids present in angiotensin. The chromatogram was uniformly sprayed with 0·1% ninhydrin in alcohol and colour was developed at 100 °C for 15 min; the spots were excised, eluted with 75% acetone and the optical density of the eluates was determined at 570 mμ (440 mμ for proline) on a Coleman Jr. model 6A spectrophotometer. The readings obtained for the amino acids applied in known concentrations served as standard recovery curves for the quantitative estimation of the amino acids present in the hydrolysates. The maximum error observed in duplicate runs of the standard mixtures was ± 5%

Biological assays

Pressor activity was assayed on the urethane-anaesthetized rat treated with hexamethonium bromide (50 mg/kg body weight intravenously) and maintained on artificial respiration. Injections were made in the external iliac vein and the blood pressure was recorded from the carotid artery with the aid of a mercury manometer of Condon's design.[8] All solutions were made isotonic before injection.

The oxytocic activity[9] was assayed on the isolated rat's uterus preparation: a virgin female albino rat weighing 150–200 g received a subcutaneous injection of stilboestrol (100 μg/kg body weight) and 15–20 hr later was killed by a blow on the head; one uterine horn was removed and suspended in an aerated 2-ml smooth muscle bath containing de Jalon's solution at 28–31 °C. The peptides to be assayed were diluted with de Jalon's solution and added to the bath in volumes not larger than 0·1 ml. The contractions of the uterine horn were recorded on a smoked drum with the aid of an isotonic frontal lever with a load on the uterus of 1·0–1·5 g and a magnification of six times.

The evaluations of pressor and oxytocic potencies of all the peptides were made by four-point assays against standard solutions of synthetic angiotensin: two doses of each substance, bearing the same higher-to-lower-dose ratio, were administered in three or four groups of four doses, each dose being given once in each group. The heights of the recorded rises in blood pressure or uterine contractions were measured and the data were treated in the manner described by Schild[10] for obtaining the potency ratio and its fiducial limits. For each comparison, two or more assays were made and the separate estimates of the logarithm of the relative potency were combined by calculating their "unweighted" mean.[11] The relative pressor or oxytocic potency of each peptide was defined as the activity of one mole of peptide in relation to the value 100 arbitrarily assigned to the activity of one mole of angiotensin.

RESULTS

Isolation of the valyl hexapeptide

Three components were separated by paper chromatography of the tryptic hydrolysate of angiotensinamide (Fig. 1). The R_f, the quantitative amino acid composition and the pressor and oxytocic potencies of component T_2 indicated that it was residual angiotensinamide not attacked by trypsin, amounting to 18 per cent of the total present in the incubation mixture. Peptides T_1 and T_3 were obtained in a molar yield of 72 and 61 per cent, respectively, and the determination of their amino acid composition

showed that T_1 contained only aspartic acid (asparagine) and arginine, and T_3 had all the amino acids of the valyl hexapeptide in the expected molar ratios.

Isolation of the arginyl heptapeptide

Paper ionophoresis of the residue from the aqueous phase of the phenylisothiocyanate degradation of angiotensinamide, yielded one major component that moved towards the cathode with a mobility that was roughly estimated to correspond to 1·5 positive charges, by comparison with the mobility of lysine. Chromatography of

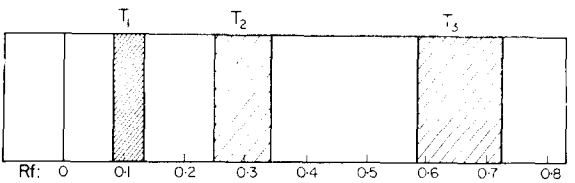

FIG. 1. Diagram showing the resolution of the tryptic hydrolysate of angiotensinamide by paper chromatography. Solvent system, butanol–acetic acid–water (63 : 10 : 27, by volume).

this material with butanol–acetic acid–water yielded a single component with an R_f of 0·42. Hydrolysis and determination of its amino acid composition indicated that it was the expected arginyl heptapeptide, in a molar yield of 17 per cent.

Biological activity

The relative pressor and oxytocic potencies found for the five peptides that were studied are shown on Table 1. The valyl hexapeptide was found to be inactive, in assays that would have detected a relative pressor potency of 1 and a relative oxytocic potency of 0·1. All the other peptides had oxytocic and pressor effects that were qualitatively indistinguishable from those previously described for angiotensinamide.[1] No significant deviation from parallelism between the log dose–response lines for angiotensin and those for each of the different peptides was found in any of the assays ($P > 0{\cdot}2$). The errors of the biological assays ranged between 5 and 15 per cent at $P = 0{\cdot}05$.

DISCUSSION

Oxytocic activity

The results of the biological assays of angiotensinamide (Table 1) show that the blocking of the γ-carboxyl group of angiotensin by an amide bond resulted in a decrease of 73 per cent in its oxytocic potency. The same degree of inactivation was observed when the aspartyl residue was substituted by a glycyl residue (78 per cent) or when it was altogether absent (77 per cent). This indicates that the aspartyl residue of angiotensin, though not essential, contributes to a marked degree for its oxytocic activity, and that this is due to the presence of the free γ-carboxyl group.

Pressor activity

Table 1 shows that the removal of the N-terminal residue of angiotensin (arginyl heptapeptide) produced a loss of pressor activity that paralleled that of oxytocic activity. This indicates that the aspartyl group of angiotensin is as important for the pressor as for the oxytocic activity. However, the presence of the free γ-carboxyl

group does not appear to be as necessary for the pressor as it is for the oxytocic activity, since the glycyl octapeptide was a more potent pressor substance than was the arginyl heptapeptide, and angiotensinamide was even more active in that respect.

The ratio of pressor to oxytocic potency was significantly greater for angiotensinamide and for the glycyl octapeptide than it was for angiotensin. This finding suggests

TABLE 1. RELATIVE PRESSOR AND OXYTOCIC POTENCIES OF SYNTHETIC ANGIOTENSIN II AND FOUR PEPTIDE ANALOGUES

		Oxytocic activity*	Pressor activity*
Angiotensin	(Asp.Arg.Val.Tyr.Val.His.Pro.Phe)	100	100
Angiotensinamide	(Asp(NH_2).Arg.Val.Tyr.Val.His.Pro.Phe)	27 (±3)	53 (±4)
Glycyl octapeptide	(Gly.Arg.Val.Tyr.Val.His.Pro.Phe)	22 (±2)	36 (±6)
Arginyl heptapeptide	(Arg.Val.Tyr.Val.His.Pro.Phe)	23 (±3)	26 (±4)
Valyl hexapeptide	(Val.Tyr.Val.His.Pro.Phe)	<0·1	<1

* Pressor and oxytocic activities are expressed by the relative potencies calculated on a molar basis the value 100 being arbitrarily assigned to angiotensin. Figures inside brackets are the fiducial limits at $P = 0{\cdot}05$.

an explanation for the greater persistence of oxytocic, relative to pressor activity, observed[1] during the first hours of partial acid hydrolysis of angiotensinamide: the hydrolysis of the amide bond would unmask the γ-carboxyl group with a consequent increase in oxytocic potency approximately twice as large as the increase in pressor potency.

The absence of detectable pressor and oxytocic activities in the valyl hexapeptide corroborates the previous indications[1, 12] that the aspartyl-arginyl portion of the angiotensin molecule is essential for both activities.

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