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DOSE-RESPONSE CURVES FOR ANGIOTENSIN II AND SYNTHETIC ANALOGUES IN THREE TYPES OF SMOOTH MUSCLE: EXISTENCE OF DIFFERENT FORMS OF RECEPTOR SITES FOR ANGIOTENSIN II

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1 The dose-response curves for angiotensin II and four analogues in rat colon and uterus, and in rabbit aorta, were obtained.

2 The ED_{50} values of angiotensin II as well as the ED_{50}s and intrinsic activities of angiotensin analogues varied between the three organs.

3 The interaction of two competitive antagonists with angiotensin II receptors was also examined. The K_I values obtained with both antagonists on the uterus differed from the values on the other tissues.

4 It is concluded that angiotensin II receptors may be different in the three organs examined.

Introduction

The stimulant effect of angiotensin and various analogues on different smooth muscle preparations shows quantitative discrepancies (Meyer, Papadimitriou & Worcel, 1970). The concentration of angiotensin giving a 50% maximal contraction varies from muscle to muscle, as well as the ratio of activity of angiotensin and its synthetic analogues. In this paper the action of angiotensin and of its analogues, agonists as well as antagonists, on rabbit aorta, rat uterus and rat colon, is further analyzed. The results obtained are interpreted as evidence for the existence of different angiotensin receptors in the organs studied.

Methods

Preparation of rabbit aorta

Rabbits of either sex weighing 1.8-3.0 kg were killed by the intravenous injection of air. The thoracic aorta was immediately dissected and excised. The organ was submerged in a Petri dish filled with McIlwain solution (mM) : NaCl 134.4, KCl 5.4, KH_2PO_4 1.34, $MgSO_4 7H_2O$ 1.34, $CaCl_2$ 1.34, glucose 13 and Na_2HPO_4 10.4, adjusted to pH 7.4 with NaOH or HCl. Perivascular tissue was cleaned from the aorta, and the latter cut into helical strips 2 mm wide (cutting angle 15°). Strips 4 cm long were mounted in a 10 ml organ bath maintained at a temperature of 37 ± 0.1°C, and bubbled with O_2. Each strip was attached to an isotonic lever giving a load of 4 g and a 4-fold magnification. The movements were recorded on a smoked drum.

Preparation of rat colon

Male or female Wistar rats (150-300 g weight) were killed by a blow on the head, and a 3 cm segment of ascending colon was excised. This section was mounted in a 10 ml chamber as in preceding experiments. In order to reduce the spontaneous activity, a solution with low Ca content was used (mM): $CaCl_2$ 0.54, NaCl 102, KCl 4.66, KH_2PO_4 1.2, $MgSO_4$ 2.44, $NaHCO_3$ 10.5, glucose 11.0, $NaHPO_4$ 2.3 and NaH_2PO_4 0.92; pH 7.4; equilibrated with 95% O_2 and 5% CO_2. The temperature of the bath was set at 30 ± 0.1°C. The organs were attached to isotonic levers that provided a 4-fold magnification and a load of 2 g, and the same recording system was used as before. The experiments were started after a one hour equilibration period during which the strips relaxed to approximately 1.5 times their original length.

Preparation of rat uterus

Immature female Wistar rats (150 g weight) were killed by a blow on the head. Uterine horns were excised and mounted in a 10 ml chamber as previously. The composition of the Ringer solution, temperature, and equilibration period were the same as were used for the rat colon.

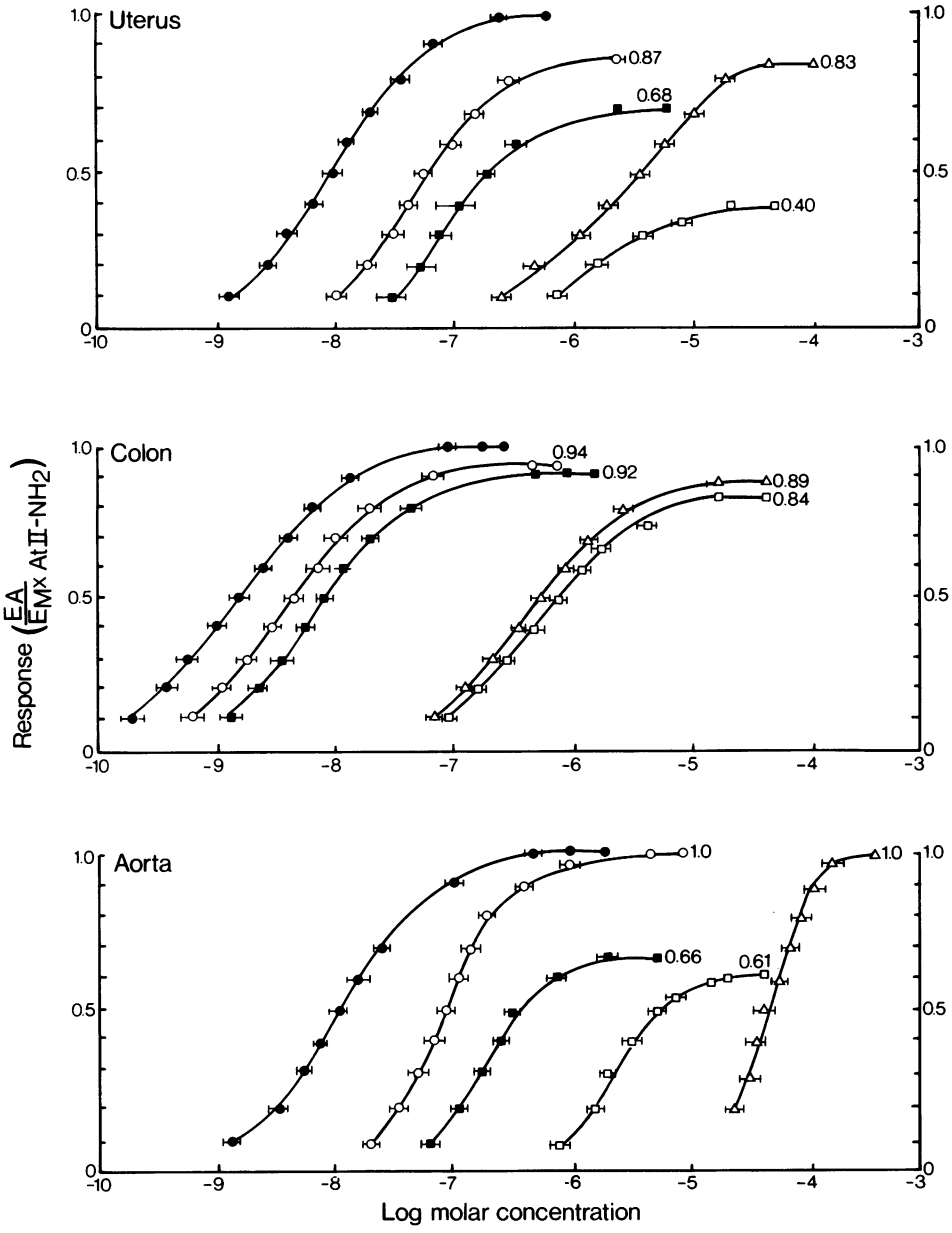

Fig. 1 Log dose-response curves to angiotensin II: AtII-NH_2 (●); [Asn^1,Diiodotyr^4,Val^5 [-angiotensin II: Dit-4-AtII-NH_2 (○); [Asn^1,Phe^4,Val^5]-angiotensin II: Phe-4-AtII (■); hexapeptide (see text): Hexa-AtII-3-8 (△); and nonapeptide (see text): Nona-AtII-NH_2 (□). This response is expressed as a fraction of the maximum effect produced by angiotensin II in each experiment. The average dose necessary to obtain a certain response was calculated. Horizontal bars show the standard errors. The curves were fitted by hand and represent the result of 18 experiments with rat uterus, 20 with rat colon and 21 with rabbit aorta in the case of angiotensin II. For the analogues, each point represents the mean of 5-8 experiments.

Exposure to drugs

The tissues were exposed to the agonists until the response reached a peak; a recovery period of at least 15 min was allowed between doses. The doses were not randomized in constructing dose-response curves.

Antagonism experiments were started after exposing the tissue to a given concentration of the inhibitor for 30 minutes.

Statistical methods

Means and standard errors were calculated and the comparisons between means were performed by methods applicable to samples of small size (Schwartz, 1960).

Drugs used

[5-Valyl]-angiotensinamide (Hypertensin, Ciba Pharmaceutical Company, Summit, N.J., U.S.A.) was purchased from Ciba. The angiotensin II analogues [Asn^1-Phe^4]-angiotensin II, hexapeptide Val-Tyr-Ileu-His-Pro-Phenylalanine, nonapeptide Asn-Arg-Val-Tyr-Tyr-Val-His-Pro-Phenylalanine and [Asn^1-$Ileu^5$-$Ileu^8$]-angiotensin II were provided by Drs F.M. Bumpus and P.A. Khairallah; [Asn^1-$Ileu^5$-Ala^8]-angiotensin II by Drs F.M. Bumpus, P.A. Khairallah and D. Regoli and [Asn^1-Diiodotyr^4-$Ileu^5$]-angiotensin II by Drs S. Fermandjian and J.L. Morgat.

Results

Dose-response curves to angiotensin and angiotensin analogues

The concentrations of angiotensin II (AtII) giving 50% maximal responses (ED_{50}) are $1.2 \pm 0.1 \times 10^{-9}$ M in rat colon (20 experiments), $1.1 \pm 0.1 \times 10^{-8}$ M in rat uterus (18 experiments) and $8.5 \pm 0.1 \times 10^{-9}$ M in rabbit aorta (21 experiments) (Figure 1). The dissimilarity of the ED_{50} values obtained could mean that the receptor sites differ. However, the ED_{50} is not necessarily equal to the real dissociation constant (Stephenson, 1956), since the relation between receptor occupancy and response may be highly non-linear (Nickerson, 1956; Ariëns, Simonis & Van Rossum, 1964). This point can be clarified either by measuring the real dissociation constant using specific irreversible antagonists (Waud, 1968), or by studying the dose-response curves of angiotensin and its structural analogues. The latter approach was used as we did not have irreversible antagonists. Experiments were performed with 4 analogues: [Asn^1, Diiodotyr^5, Val^5]-angiotensin II (DIT), [Asn^1, Phe^4, Val^5]-angiotensin II (Phe-4), the hexapeptide formed by the amino acids 3-8 of [$Ileu^5$]-angiotensin II (Hexa) and the nonapeptide Asn-Arg-Val-Tyr-Tyr-Val-His-Pro-Phe (Nonap) obtained by addition of one tyrosine residue to the [Asn^1,Val^5]-angiotensin II. The log dose-response curves are represented in Figure 1. Table 1 shows the intrinsic activities calculated as the ratio between the maximum response to the analogue and to angiotensin II; and the ratio of the ED_{50}. It is evident that there are some differences in the response of the three organs to angiotensin and its analogues, which can be explained in different ways. The results suggest that the main difference between uterus and colon is that the colon has a greater receptor reserve. In the colon, all the compounds are full agonists, or nearly so, and the curves lie further to the left, but the order of potency of the five compounds is the same. On the other hand, the hexapeptide is obviously anomalous in the aorta, the ED_{50} being 20 times higher than that of nonapeptide.

Interactions between angiotensin and [Asn^1, Phe^4]-angiotensin II

In order to obtain further evidence, attempts were made to compare the real dissociation constant for one of the partial agonists, the Phe-4 compound, in uterus and aorta. Aorta and uterus were stimulated with Phe-4 in the presence of varying concentrations of angiotensin. Figure 2 shows that angiotensin modified the dose-response curves of the analogue as would be expected from the interaction of a full agonist and a partial agonist on a common receptor site (Ariëns & de Groot, 1964). The value of the dissociation constant for the Phe-4 compound in both organs was determined by the experimental procedure described by Stephenson (1956). Let A_1,A_2 and A_3 be the concentrations of an active agonist (in this case AtII) and let P be the concentration of a partial agonist, such that A_1 produces the same effect as P and A_2 has the same action as A_3 and P given together.

If x is the proportion of receptors occupied by the partial agonist, then

$$x = 1 - \frac{A_2 - A_1}{A_3}$$

(Stephenson, 1956)

and since

$$K = \frac{P(1-x)}{x}$$

K can be calculated from the concentrations P,

Table 1 ED_{50} values, intrinsic activities, and activity ratios of angiotensin and angiotensin analogues.

	Rat uterus			*Rat colon*			*Rabbit aorta*		
	ED_{50} (M)	*Intrinsic activity*	*Activity ratio**	ED_{50} (M)	*Intrinsic activity*	*Activity ratio*	ED_{50} (M)	*Intrinsic activity*	*Activity ratio*
[Asn^1,Val^5]-angiotensin II	$1.1 \pm 0.2 \times 10^{-8}$ (18)†	1.00	1000.0	$1.2 \pm 0.2 \times 10^{-9}$ (20)	1.00	1000.0	$8.5 \pm 0.1 \times 10^{-9}$ (21)	1.00	1000.0
[Asn^1,Phe^4,Val^5]-angiotensin II	$1.8 \pm 0.4 \times 10^{-7}$ (5)	0.56	61.0	$1.0 \pm 0.2 \times 10^{-8}$ (5)	0.92	110.0	$1.7 \pm 0.2 \times 10^{-7}$ (8)	0.60	7.0
[Asn^1,Diiodotyr^4,Ileu^5]-angiotensin II	$4.7 \pm 0.3 \times 10^{-8}$ (5)	0.87	234.0	$5.0 \pm 0.3 \times 10^{-9}$ (5)	0.94	240.0	$8.6 \pm 0.1 \times 10^{-8}$ (5)	1.00	99.0
Hexa‡	$2.2 \pm 0.4 \times 10^{-6}$ (5)	0.83	5.0	$5.0 \pm 0.3 \times 10^{-7}$ (7)	0.89	2.4	$5.6 \pm 0.3 \times 10^{-5}$ (6)	1.00	0.1
Nonap‡	$2.0 \pm 0.2 \times 10^{-6}$ (5)	0.40	5.5	$6.4 \pm 0.3 \times 10^{-7}$ (5)	0.84	1.9	$2.2 \pm 0.4 \times 10^{-6}$ (5)	0.61	3.9

* Activity ratio : ratio between analogue ED_{50} and angiotensin ED_{50} x 1000; † number of experiments; ‡ see text.

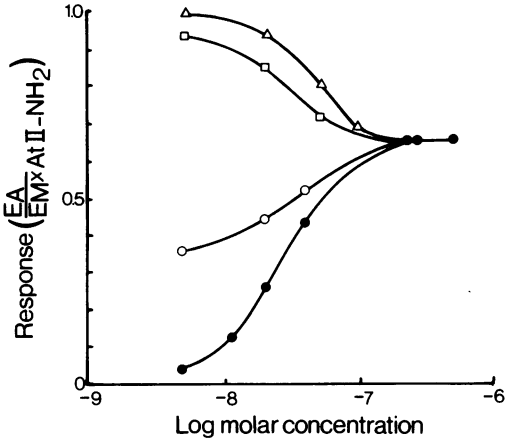

Fig. 2 Cumulative log dose-response curves of the partial agonist [Asn^1,Phe^4,Val^5]-angiotensin II in the presence of angiotensin II on the rabbit aorta.

The figure shows points from one of a series of six identical experiments. The response is given as a fraction of the maximum response to angiotensin II. The control log dose-response curve to Phe-4-AtII is shown by solid circles. The other curves were done in the presence of 1×10^{-8} M (○), 5×10^{-8} M (□) and 1×10^{-7} M (△) angiotensin II.

A_1, A_2 and A_3. The K value for Phe-4 in aorta was $2.7 \pm 0.5 \times 10^{-7}$ M (five experiments) and in uterus $8.5 \pm 2.0 \times 10^{-8}$ M (13 experiments). The difference between both values was significant ($P < 0.001$). The results are shown in Table 2.

Interactions between [Asn^1, Val^5]-angiotensin II and [Asn^1, Ileu^5, Ileu^8]-angiotensin II and [Asn^1, Ileu^5, Ala^8]-angiotensin II

As already demonstrated (Bumpus, Khairallah & Smeby, personal communication), [Asn^1, Ileu^5, Ileu^8]-angiotensin II is a competitive antagonist of angiotensin II. The drug has an antagonist action on colon, uterus and aorta. Figure 3 shows that there is a clear difference between the dissociation constants (K_I) obtained in the three organs. The K_I are $3.2 \pm 0.17 \times 10^{-9}$ M (11 experiments) in aorta, $8.2 \pm 0.11 \times 10^{-9}$ M (eight experiments) in uterus, and $1.3 \pm 0.14 \times 10^{-8}$ M (10 experiments) in colon. The differences between the three organs are significant ($P < 0.01$ between uterus and colon and $P < 0.001$ between aorta and colon and between uterus and aorta).

[Asn^1, Ileu^5, Ala^8]-angiotensin II is also a competitive antagonist of angiotensin II (Türker, Yamamoto, Khairallah & Bumpus, 1971). As in

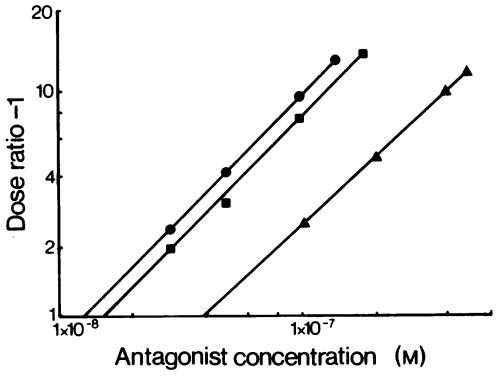

Fig. 3 Antagonism of angiotensin II by [Asn¹,Ileu⁵,Ala⁸]-angiotensin II. Logarithmic plots of (dose-ratio − 1) against antagonist concentration. Each point is the average of 8-11 experiments. The concentration at which the line cuts the abscissa (dose-ratio − 2) gives the dissociation constant for the antagonist (Schild, 1947), which is different in the three tissues studied; uterus (●), aorta (■) and colon (▲).

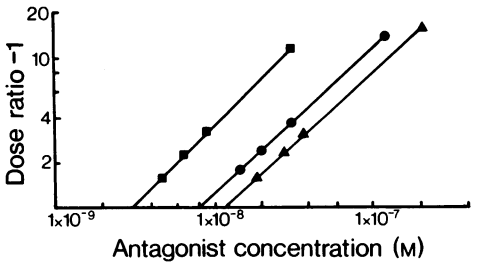

Fig. 4 Antagonism of angiotensin II by [Asn¹,Ileu⁵,Ala⁸]-angiotensin II. Data plotted as in Figure 3. Each point is the average of 8-13 experiments.

the previous experiments, the inhibitory activity of the drug was measured on the three organs (Figure 4). [Asn¹, Ileu⁵, Ala⁸]-angiotensin II has essentially the same K_I in rabbit aorta $1.3 \pm 0.17 \times 10^{-8}$ M (10 experiments) and in rat uterus 1.5 ± 0.15 (13 experiments) where the curves do not differ significantly. On the other hand, the K_I is significantly higher ($P < 0.001$) in rat colon ($3.9 \pm 0.10 \times 10^{-8}$ M, eight experiments).

The results presented above suggest dissimilarities in the steric structure of receptors since, in the case of the competitive antagonists, the dissociation constants (K_I) should be similar in different organs and with different agonists if the receptors are identical (Schild, 1957).

Discussion

The existence of differences between the ED_{50} obtained with angiotensin in the three organs tested could indicate that the steric structure of angiotensin receptors in these muscles is not the same. Nonetheless, ED_{50} cannot be considered identical to the dissociation constant K, since the dose-response curve can diverge considerably from the concentration-binding curve, due to varying

Table 2 Occupation of receptors (x) and affinity constants of [Asn¹,Phe⁴,Ileu⁵]-angiotensin II

	x	$K \times 10^{-7}$ M	x	$K \times 10^{-7}$ M
1	0.71	2.0	0.76	0.6
2	0.67	2.5	0.64	0.8
3	0.31	4.4	0.62	0.4
4	0.86	1.6	0.82	0.9
5	0.63	2.9	0.72	0.4
6			0.58	1.4
7			0.42	1.4
8			0.64	0.4
9			0.44	1.1
10			0.76	0.6
11			0.81	0.5
12			0.80	1.2
13			0.75	1.1
Mean		2.7		0.8
s.e.		± 0.5		± 0.1
$\frac{t}{P}$		5.69 <0.001		

causes of non-linearity of the occupancy-response relationship (Stephenson, 1956; Waud, 1968). The dose-response curves to AtII and analogues suggest that the main difference between uterus and colon is that colon has a greater receptor reserve than uterus (Ariëns *et al.*, 1964). On the other hand, the position of the dose-response curve to the hexapeptide is clearly anomalous in aorta. The values of the dissociation constants K of the partial agonist [Asn^1, Phe^4]-angiotensin II suggest even more strongly a difference between the angiotensin receptors in uterus and aorta.

Competitive antagonists are more useful than agonists for the identification of receptors since their action does not involve other steps beyond the occupation of receptor sites (Waud, 1968). Both antagonists used ([Asn^1, $Ileu^5$, $Ileu^8$]-angiotensin II and [Asn^1, $Ileu^5$, Ala^8]-angiotensin II) appear to act competitively since the pA_2-pA_{10} ratio is about 0.95 for both (Schild, 1957) in all three organs. The results obtained with both antagonists show a different picture from that obtained with the agonists. The K_I values for [Asn^1, $Ileu^5$, $Ileu^8$]-angiotensin II are different in aorta, uterus and colon; with [Asn^1, $Ileu^5$, Ala^8]-angiotensin II, on the other hand, uterus and aorta give practically the same K_I, which differs from the value in colon. According to these results, the steric conformation of angiotensin receptors could be different in the three organs.

Our findings are in accord with those of Bumpus (1971) who studied the actions of a series of angiotensin analogues on the blood pressure of the vagotomized and nephrectomized rat and on the contraction of rat uterine smooth muscle. As in our case, he observed marked dissimilarities in the way the analogues affected each of the preparations. He suggested that the results observed could be due to differences in the structure of receptor sites or in their environment.

It is interesting to review some recent publications on the subject of structure-activity relationships among angiotensin analogues. Regoli & Park (1971) studied the actions of numerous analogues of angiotensin I and II on rat blood pressure and isolated colon and stomach. Among these compounds, three of them showed very different activities in the systems tested. These angiotensin II analogues are [1-aminocyclopentone carbonylic acid)-7]-angiotensin II and [Phe^4, $Ileu^5$, Tyr^8]-angiotensin II. Marshall, Vine & Needleman (1970) observed that the competitive antagonist, [Phe^4, $Ileu^5$, Tyr^8]-angiotensin II, has much higher blocking action on rat blood pressure than on uterine smooth muscle. Peach, Bumpus & Khairallah (1971) studied the release of catecholamines from cat adrenal medulla induced by angiotensin I and II and various analogues. These authors reported a very marked stimulation of catecholamine release by angiotensin I. This peptide has 1/20th of the potency of angiotensin II on blood pressure stimulation or oxytocic tests but it is essentially as potent as angiotensin II in adrenal medulla. On the other hand, [Asn^1, $Ileu^5$, Ala^8]-angiotensin II, a competitive antagonist of angiotensin II in the three organs we tested, has a marked (20% of angiotensin II) agonist action on adrenal medulla.

In conclusion, the examination of the effects of angiotensin II and its synthetic analogues (agonists as well as antagonists) on different organs, suggests that angiotensin II receptor sites in different tissues do not have a uniform structure.

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