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Characterization of angiotensin receptors in vascular and intestinal smooth muscles

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Summary

1. A series of analogues of angiotensin II (AT_{II}) has been used in the present experiments to characterize receptors for AT_{II} in intestinal (rat stomach strip, rat colon) and vascular (rabbit aorta) smooth muscles. Two types of compounds have been chosen: (a) agonists with reduced potency, in which 4-Tyr, 6-His or 7-Pro had been substituted with L-Ala or with Gly and 1-aminocyclopentane carboxylic acid (Acpc) and (b) competitive antagonists (8-Gly-AT_{II}, 8-Leu-AT_{II}).

2. Replacement of 4-Tyr, 6-His and 7-Pro with L-Ala decreases the potency, but does not influence the maximum effect of the analogue, while substitution of the same residue with Gly and Acpc reduces both potency and maximum effect.

3. Compounds showing full size maximum responses were chosen to establish the following order of potency on the three preparations: AT_{II}>4-Phe-AT_{II}>7-Ala-AT_{II}>6-Ala-AT_{II}>4-Ala-AT_{II}.

4. The four derivatives of AT_{II} were completely inactive on tissues desensitized with AT_{II}. The responses to 5-hydroxytryptamine, acetylcholine and noradrenaline were not significantly modified, except for the rat colon.

5. pA_2 values for the two competitive antagonists against AT_{II} and 4-Phe-AT_{II} were estimated in the three preparations by the use of the cascade superfusion technique. For comparison, pA_2 values were also estimated in rat stomach strips and rabbit aortas suspended in a normal organ bath, according to the method of Schild (1947). The similarities of the pA_2 values obtained in two series of experiments indicate that (a) the cascade superfusion technique is suitable for this type of study and (b) the receptor for AT_{II} in the three tissues may be the same.

6. It is suggested that receptors for AT_{II} in intestinal and vascular smooth muscles may be the same, because (a) the order of potency of various agonists follows the same pattern, (b) the agonists are inactive on tissues desensitized with AT_{II}, (c) pA_2 values for competitive antagonists are similar in the three preparations.

Introduction

Recent studies have shown that analogues of angiotensin II (AT_{II}) in which one or more of the 8 amino acids has been substituted show modified biological activity (Regoli & Park, 1972). In particular, substitution of 8-Phe with aliphatic amino acids results in specific competitive antagonists of angiotensin (Pals, Masucci,

Sipos & Denning, 1971 ; Regoli, Park, Rioux & Chan, 1971 ; Türker, Yamamoto, Khairallah & Bumpus, 1971).

The availability of these analogues of angiotensin allows the properties of angiotensin receptors in different tissues to be compared in the following ways (Schild, 1973):

1. By determining the order of potency of different agonists in each tissue.
2. By estimating pA_2 values for antagonists in each tissue.
3. By testing the effect of desensitization to angiotensin on the sensitivity of the tissue to other analogues.

These tests have been applied in the present study to characterize angiotensin receptors in intestinal smooth muscle (rat stomach strip, rat colon) and in vascular smooth muscle (rabbit thoracic aorta).

This study required the synthesis and the pharmacological characterization of several new compounds structurally related to angiotensin.

Methods

The effects of several angiotensin II (AT_{II}) analogues have been tested *in vivo* on the rat blood pressure and *in vitro* on three isolated organs, the rat stomach strip (Vane, 1957), the rat ascending colon (Regoli & Vane, 1964) and the rabbit thoracic aorta (Furchgott & Bhadrakom, 1953).

The *in vivo* experiments were performed on albino rats weighing 250–300 g. The animals were nephrectomized under ether anaesthesia 24 h before and anaesthetized with 1·4 g/kg of urethane (s.c.) on the day of the experiment. The pressor activity of analogues of AT_{II} was compared with that of AT_{II} ; analogues of AT_{II} with pressor activity were also tested for antagonism by administering into the tail vein (at the rate of 0·05 ml/min) for at least half an hour, a dose just insufficient to raise the blood pressure. Standard doses of AT_{II} were injected before, 10 min after starting the intravenous infusion of the analogue and 30 to 35 min after stopping the infusion.

Most of the experiments *in vitro* were performed with the cascade superfusion technique of Vane (1964), using three homologous tissues in each experiment. Oxygenated (95% O_2 and 5% CO_2) Krebs solution at 37° C was perfused at the rate of 10 ml/min, and the peptides were injected with a Harvard pump at the rate of 0·1 ml/min into the superfusing fluid for 3–5 min on the rat stomach strip, 1–3 min on the rat colon and 5–8 min on the rabbit aorta. A period of 20 min was allowed between two doses. The Krebs had the following composition (mM): NaCl, 118 ; $MgSO_4 \cdot 7H_2O$, 1·18 ; KH_2PO_4, 1·18 ; glucose, 5·55 ; $NaHCO_3$, 25·0 ; $CaCl_2 \cdot 6H_2O$, 2·5 and KCl, 4·70.

The contractions of the tissue were recorded with an isotonic transducer (Harvard No. 356) at a tension of 1·5–2·0 grams. Maximum contractions were elicited at the beginning of each experiment with 10^{-7}M AT_{II} on the rat stomach strip and the rabbit aorta, and with $2{\cdot}3 \times 10^{-8}$M on the rat colon. Thereafter, dose-response curves to AT_{II} and analogues were recorded. Single large doses of analogues were applied, in some experiments, to measure the maximum effect. In general, dose-response curves were measured with an interrupted rather than a cumulative dose sequence.

Analogues were tested for antagonism against AT_{II} by infusion of the peptide at doses which did not produce any contraction, for at least 10 min before and during the administration of the test doses of AT_{II}.

In a second series of experiments, the three tissues were desensitized by infusing AT_{II} 10^{-7} or 10^{-6}M. After an initial maximum contraction, the baseline of the tissues came back to the control level; at this point, some of the analogues as well as 5-hydroxytryptamine, acetylcholine or noradrenaline were tested at submaximal doses.

In a third series of experiments, pA_2 values for 8-Gly-AT_{II} on the three preparations were measured with AT_{II} or 4-Phe-AT_{II} as agonists. 8-Leu-AT_{II} was also tested against AT_{II} on the rat stomach strip and rabbit aorta. The analysis was done by the method of Arunlakshana & Schild (1959).

When high doses (10^{-6}M) of AT_{II} were applied, rat stomach strips showed some desensitization, though this effect was less evident on rabbit aorta and rat colon. To minimize this effect, only one dose of AT_{II} higher than 10^{-7}M was tested in the presence of the antagonist (8-Gly-AT_{II} or 8-Leu-AT_{II}) in each experiment.

The same antagonists were used to estimate pA_2, according to the method of Schild (1947). pA_2 values of AT_{II}/8-Gly-AT_{II} and AT_{II}/8-Leu-AT_{II} were measured by using strips of rat stomach and rabbit aorta suspended in a 10 ml bath, containing Krebs solution at 37° C. Assays were carried out simultaneously on two strips and, in most experiments, the two strips were derived from the same animal. The resting tension was 1·5 g and changes of tension were detected with a Grass force displacement transducer (FT-03-C) on a Grass polygraph (model No. 79).

Antagonists were injected into the bath and maintained in contact with the tissue for 5 or 15 min before application of AT_{II} at doses twice as great as the control doses. After the antagonist was washed out, the response to the control dose of AT_{II} was tested 2 or 3 times in order to make sure that recovery was complete at the time the second dose of antagonist was applied. Each preparation was used to determine pA_2 after 5 and 15 min of contact and for one antagonist only.

TABLE 1. *Analogues of angiotensin*

1 H-Asp	2 — Arg	3 — Val	4 — Tyr	5 — Ile	6 — His	7 — Pro	8 — Phe	(AT_{II})
Analogues substituted in position 4.								
—	—	—	Phe	—	—	—	—	(4-Phe-AT_{II})*
—	—	—	Acpc**	—	—	—	—	(4-Acpc-AT_{II})[a]
—	—	—	Ala	—	—	—	—	(4-Ala-AT_{II})[b]
—	—	—	Gly	—	—	—	—	(4-Gly-AT_{II})*
Analogues substituted in position 6.								
—	—	—	—	—	Acpc	—	—	(6-Acpc-AT_{II})[a]
—	—	—	—	—	Ala	—	—	(6-Ala-AT_{II})[c]
—	—	—	—	—	Gly	—	—	(6-Gly-AT_{II})*
Analogues substituted in position 7.								
—	—	—	—	—	—	Acpc	—	(7-Acpc-AT_{II})[a]
—	—	—	—	—	—	Ala	—	(7-Ala-AT_{II})[d]
—	—	—	—	—	—	Gly	—	(7-Gly-AT_{II})*

*Newly synthesized peptides
**Acpc (1-aminocyclopentane carboxylic acid)
([a]) Park, Asselin & Berlinguet, 1970
([b]) Seu, Smeby & Bumpus, 1962a
([c]) Park, W. K., Seu, J. H., Smeby, R. R. & Bumpus, F. M., to be published.
([d]) Seu, Smeby & Bumpus, 1962b

All compounds, shown in Table 1, as well as 8-Gly-AT_{II} and 8-Leu-AT_{II} were synthesized in our laboratory with the solid-phase method of Merrifield (1963) using the reaction vessel described by Park & Regoli (1972). Details of the synthesis and purification of the newly synthesized peptides are given in the appendix. All peptides were dissolved in 0·9% w/v NaCl solution (saline) at a concentration of 10^{-3} to 5×10^{-3}M and the concentrated solutions were kept at −20° C for a maximum of two weeks. From these solutions, fresh dilutions were made each day in Krebs solution. Concentrations of peptides are given in mol/litre. 5-Hydroxytryptamine (5-hydroxytryptamine creatinine sulphate, Sigma) acetylcholine (acetylcholine chloride, Roche) and noradrenaline (noradrenaline hydrochloride, Sigma) were dissolved in distilled water; they were stored and diluted in the same way as the peptides.

The results are expressed as means ± S.E.; results were compared by means of Student's *t* test for paired data.

Results

Effects of angiotensin analogues on the rat blood pressure

As reported by Regoli & Park (1972), the increase of the blood pressure in nephrectomized rats anaesthetized with urethane is linearly related to the log of the dose of angiotensin, provided the increase does not exceed 50 mmHg. It is therefore possible to compare the potency of angiotensin analogues *in vivo*.

Results obtained with AT_{II} and analogues substituted in positions 4, 6 and 7 are shown in Figure 1.

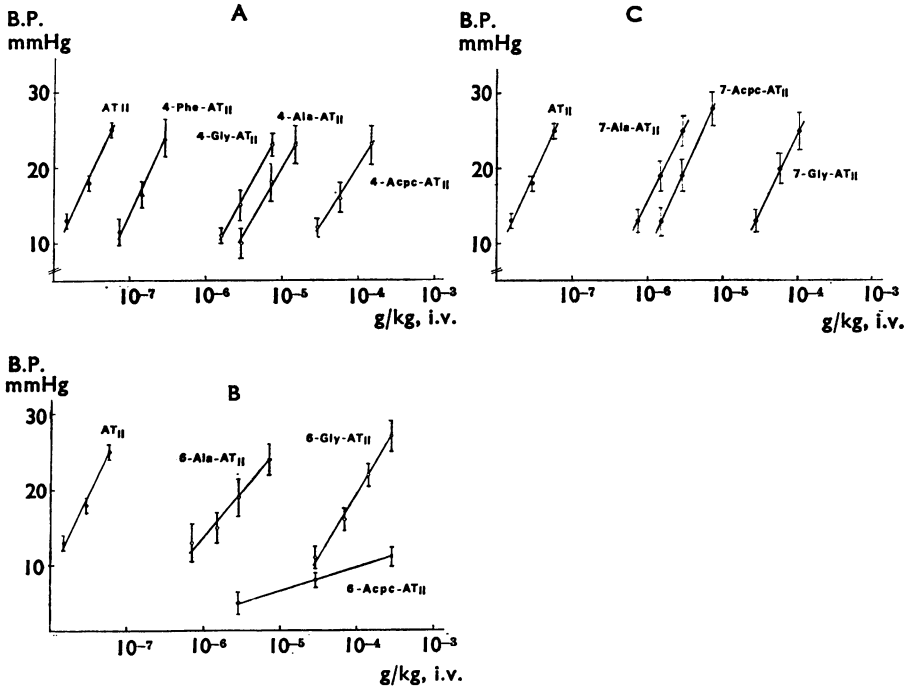

FIG. 1. Dose-response curves of angiotensin II (AT_{II}) and analogues substituted in position 4 (A), 6 (B) and 7 (C) on the blood pressure of nephrectomized rats, anaesthetized with urethane. Points indicate means of 8 experiments and vertical bars S.E. Abscissae: Concentration of AT_{II} and analogues in g/kg.

All compounds with the exception of 6-Acpc-AT_{II} gave dose-response curves parallel to that of AT_{II}. When tested for antagonism, no inhibition was observed with any of the analogues, except for a slight effect with 4-Phe-AT_{II}. The relative pressor activity of the angiotensin analogues, as well as the range of the effective doses, are shown in Table 2.

TABLE 2. *Pressor activity (%) of various analogues of angiotensin II (AT_{II}) substituted in positions 4, 6 and 7, as determined in nephrectomized rats anaesthetized with urethane*

Compound	Pressor activity %	Range of effective doses (g/kg)
AT_{II}	100	$2{\cdot}5-7{\cdot}5\times10^{-8}$
4-Phe-AT_{II}	20	$8{\cdot}5\times10^{-8}-5\times10^{-7}$
4-Acpc-AT_{II}	0·05	$5\times10^{-5}-2{\cdot}5\times10^{-4}$
4-Ala-AT_{II}	0·51 (0·31)[a]	$5\times10^{-6}-7{\cdot}5\times10^{-5}$
4-Gly-AT_{II}	0·74	$2{\cdot}5-8{\cdot}5\times10^{-6}$
6-Acpc-AT_{II}	<0·001	$5{\cdot}0\times10^{-6}-5\times10^{-4}$
6-Ala-AT_{II}	1 (0·8)[b]	$8{\cdot}5\times10^{-7}-8{\cdot}5\times10^{-6}$
6-Gly-AT_{II}	0·05	$5{\cdot}0\times10^{-5}-5{\cdot}0\times10^{-4}$
7-Acpc-AT_{II}	1	$2{\cdot}5\times10^{-6}-8{\cdot}5\times10^{-6}$
7-Ala-AT_{II}	2·5 (0)[b] (0)[c]	$8{\cdot}5\times10^{-7}-5{\cdot}0\times10^{-6}$
7-Gly-AT_{II}	0·06	$5{\cdot}0\times10^{-5}-1{\cdot}0\times10^{-4}$

In parentheses, the values previously reported by other investigators.
([a]) Seu *et al.*, 1962b.
([b]) Khairallah *et al.*, 1970
([c]) Page & Bumpus, 1961

Several of these compounds have been tested previously on the rat blood pressure by other investigators. The results obtained in the present experiment confirm generally those reported by Seu, Smeby & Bumpus (1962b) for 4-Ala-AT_{II} and by Khairallah, Toth & Bumpus (1970) for 6-Ala-AT_{II}. 7-Ala-AT_{II}, however, possessed 2·5% of the pressor activity of AT_{II} in our experiments whereas the same compound was reported to be inactive by Page & Bumpus (1961) and Khairallah *et al.* (1970).

Effect of angiotensin analogues on the rat stomach strip, the rat colon and the rabbit aorta

Dose-response curves of AT_{II} and analogues on the rat stomach strip are shown in Figure 2. The action of analogues substituted in position 4 (Fig. 1A), in position 6 (Fig. 1B) and in position 7 (Fig. 1C) confirms in general the results obtained *in vivo* and shows that the replacement of 4-Tyr with Phe brings about a large reduction in potency, but does not influence the slope or the maximum of the log-concentration-effect curve. A much greater decrease of potency is observed with 4-Ala-AT_{II}, 4-Acpc-AT_{II} and 4-Gly-AT_{II}. These compounds have approximately the same potency (see pD_2 value in Table 3) but the maximum response is slightly reduced, especially with 4-Gly-AT_{II}. These results indicate that the maximum potency requires a phenolic group in position 4. The absence of the hydroxyl group of Tyr reduces the potency by a factor of 12 and the absence of the phenolic group reduces it by a factor of 1,200. A maximal response equal to that of AT_{II} is obtained so long as an alpha asymmetric carbon is present in the peptide chain (4-Ala-AT_{II}), but it is slightly reduced when amino acids, such as Gly or Acpc are used to replace 4-Tyr.

The importance of 6-His for the action of AT_{II} is indirectly evaluated by the results of the experiment shown in Figure 2B. Replacement of 6-His with Ala reduces the potency by a factor of 1,000 without modifying significantly the maxi-

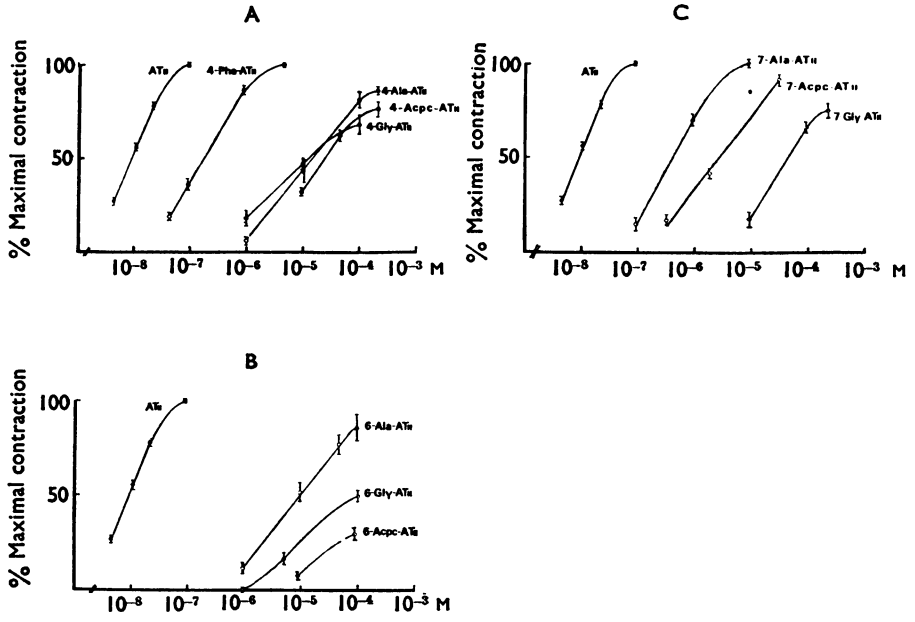

FIG. 2. Dose-response curves of angiotensin II (AT_{II}) and analogues substituted in positions 4 (A), 6 (B) and 7 (C) on the rat isolated stomach strip, suspended in a cascade superfusion system. Points indicate the means of 6–9 values and vertical bars, the S.E. Abscissae: Molar concentration of AT_{II} analogues.

mum response obtainable; replacement with Gly or Acpc brings about a marked decrease of potency as well as a reduction of the maximum response. Maximum contractions elicited by 6-Gly and 6-Acpc-AT_{II} are 50% or less of the maximum obtained with AT_{II}.

The reduction of potency in analogues substituted in position 7 is much less marked than in those substituted in positions 4 and 6. Substitution of Pro with Ala reduces the potency by a factor of about 15 without modifying the maximum response. A loss of potency as well as a slight decrease of the maximum effects are observed with 7-Acpc-AT_{II} and 7-Gly-AT_{II}.

The results shown in Fig. 2 suggest that when a normal constituent of AT_{II} is replaced by Ala the most important effect is a loss of potency. Replacement of the same residue with Gly or Acpc, however, brings about in addition a decrease of the maximal response.

To estimate the order of potency of AT_{II} and analogues on the two other preparations (rabbit aorta and rat colon), we used 4-Phe-AT_{II} and the three Ala derivatives, all of which produced equal maximal responses. The results are summarized in Fig. 3 and Table 3.

The first important difference between the three tissues is the sensitivity to AT_{II}; the most sensitive tissue is the rat colon, followed by the rabbit aorta and by the rat stomach strip. The order of potency of the 4 analogues of AT_{II} in the three preparations is the same as that observed *in vivo*, namely: AT_{II}>4-Phe-AT_{II}> 7-Ala-AT_{II}>6-Ala-AT_{II}>4-Ala-AT_{II}.

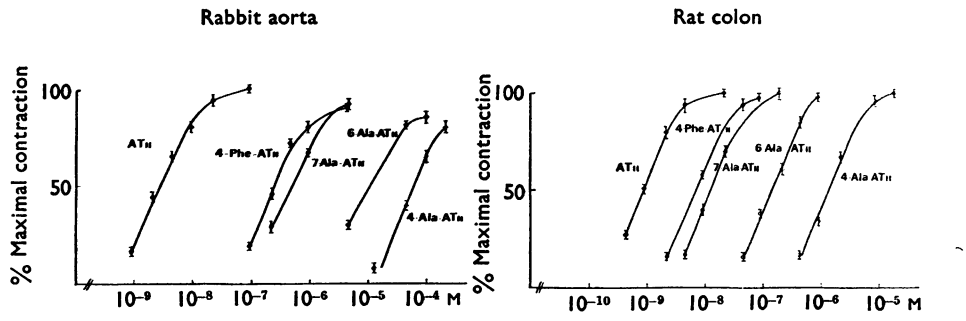

FIG. 3. Dose-response curves of angiotensin II (AT_{II}) and analogues on the rabbit aorta (on the left) and on the rat colon (on the right). Same explanations as for Figure 2.

TABLE 3. *Maximum response (MR %) and potency (pD_2) of several analogues of angiotensin II (AT_{II}) substituted in positions 4, 6 and 7, as determined on the isolated rat stomach strip, rat colon and rabbit aorta*

Compound	Rat stomach MR%	pD_2	Rel. pot.	Rat colon MR%	pD_2	Rel. pot.	Rabbit aorta MR%	pD_2	Rel. pot
AT_{II}	100	8·0	1,000	100	9·05	1,000	100	8·53	1,000
4-Phe-AT_{II}	100	6·8	60	100	8·16	135	90	6·60	11
4-Acpc-AT_{II}	80	4·7	0·8	—	—	—	—	—	—
4-Ala-AT_{II}	90	4·8	0·6	100	5·88	0·78	80	4·20	0·1
4-Gly-AT_{II}	70	4·8	0·6	—	—	—	—	—	—
6-Acpc-AT_{II}	30	—	—	—	—	—	—	—	—
6-Ala-AT_{II}	90	5·0	1·0	100	6·83	6·8	90	4·97	0·27
6-Gly-AT_{II}	50	4·0	0·1	80	5·7	0·6	7	—	—
7-Acpc-AT_{II}	90	5·8	6·0	—	—	—	—	—	—
7-Ala-AT_{II}	100	6·4	2·4	100	7·92	80	90	6·3	6
7-Gly-AT_{II}	70	4·4	0·25	—	—	—	—	—	—

pD_2 is the negative logarithm of the molar concentration of the agonist which gives 50% of the maximal response.

The relative potencies (see Table 3) show some variation from one tissue to another. The same compounds were tested on the three tissues after desensitization by continuous infusion of AT_{II}, 10^{-7}M for the rat stomach strip and the rat colon and 10^{-6}M for the rabbit aorta. This dose of AT_{II} caused a contraction of the tissues which gradually diminished and returned to the control level after approximately 1–2 h, despite the infusion of AT_{II}. The three preparations were then insensitive to test doses of AT_{II} and to doses of the analogues which produced 50% or more of the maximum response in non-desensitized tissues. The effects of acetylcholine and 5-hydroxytryptamine on the rat stomach strip, as well as the effects of 5-hydroxytryptamine and noradrenaline on the rabbit aorta were not significantly modified. A slight decrease of the response to both acetylcholine and 5-hydroxytryptamine was observed on the rat colon (Table 4).

Estimation of pA_2 for comparison of angiotensin II receptors

In order to compare the properties of the receptors in different tissues, experiments were undertaken to estimate pA_2 for two antagonists of AT_{II}. The antagonists were tested against two agonists (AT_{II} and 4-Phe-AT_{II}), in order to determine if they produced similar pA_2 values. 8-Gly-AT_{II} and 8-Leu-AT_{II} have recently been shown to be specific and competitive antagonists of AT_{II} on the rat stomach strip (Rioux, Park & Regoli, 1973).

TABLE 4. *Effects of several angiotensin II analogues, 5-hydroxytryptamine (5-HT), acetylcholine (ACh) and noradrenaline (NA), in absence and in presence of a desensitizing dose of* AT_{II} (1×10^{-7}M). *Analogues of* AT_{II} *were also tested after recovery for 1 hour. Results are given as the % of maximum contraction (mean*±S.E. *of 5 experiments)*

Compound	Dose	No desensitization	Desensitization	Approximately 1 h after desensitization
Rat stomach strip				
4-Phe-AT_{II}	2×10^{-7}M	50±4	0	45±4
4-Ala-AT_{II}	2×10^{-5}M	43±4	0	25±2
6-Ala-AT_{II}	1×10^{-5}M	52±5	0	56±5
7-Ala-AT_{II}	5×10^{-7}M	43±3	0	50±2
5-HT	$2{\cdot}9\times10^{-9}$M	67±4	64±7	—
ACh	$1{\cdot}3\times10^{-7}$M	72±3	63±6	—
Rabbit aorta				
4-Phe-AT_{II}	$2{\cdot}5\times10^{-7}$M	40±3	0	30±3
4-Ala-AT_{II}	5×10^{-5}M	41±3	0	37±3
6-Ala-AT_{II}	1×10^{-5}M	60±4	0	48±4
7-Ala-AT_{II}	5×10^{-7}M	35±2	0	33±3
5-HT	$1{\cdot}5\times10^{-7}$M	38±2	34±6	—
NA	$2{\cdot}8\times10^{-8}$M	47±3	38±5	—
Rat colon				
4-Phe-AT_{II}	2×10^{-7}M	72±4	0	25±2
4-Ala-AT_{II}	$2{\cdot}5\times10^{-6}$M	67±3	0	45±3
6-Ala-AT_{II}	1×10^{-6}M	94±4	0	65±3
7-Ala-AT_{II}	1×10^{-7}M	92±4	0	45±2
5-HT	$1{\cdot}7\times10^{-6}$M	70±6	23±9*	—
ACh	$5{\cdot}2\times10^{-7}$M	64±4	47±2*	—

*$P < 0{\cdot}05$

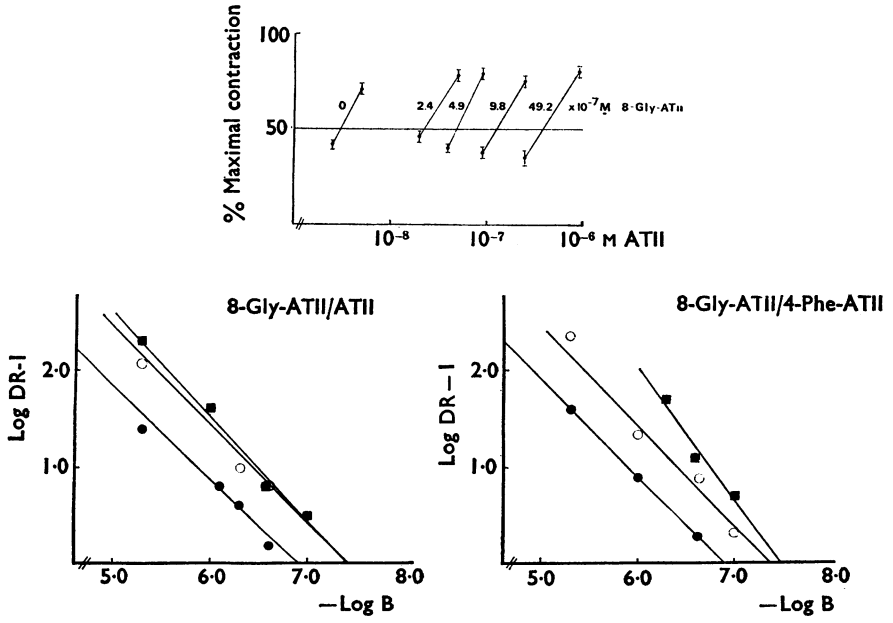

FIG. 4. Above: Effect of angiotensin II (AT_{II}) in absence and in presence of 8-Gly-AT_{II} on rabbit aorta. Means±S.E. of 6 experiments. Abscissae: Molar (M) concentration of AT_{II}. (This is an example of the experimental protocol used to obtain the data for the log-log plots shown in the two sections below.) Below: Plots of log dose-ratio−1 (Log DR−1) against antagonist concentration (−Log B), showing competitive antagonism of AT_{II}/8-Gly-AT_{II} (left) on the three tissues and of 4-Phe-AT_{II}/8-Gly-AT_{II} (right) on rat stomach strip and rabbit aorta. In each instance, except 4-Phe-AT_{II}/8-Gly-AT_{II} on the rat colon, the slope is close to unity. ●——●, Rat stomach strip; ○——○, rabbit aorta; ■——■, rat colon.

TABLE 5. *pA_2 values of* 8-*Gly-AT_{II} and* 8-*Leu-AT_{II} against AT_{II} and* 4-*Phe AT_{II}*

Cascade superfusion technique (isotonic recording)
(pA_2 values have been estimated from data shown in Fig. 4)

Tissue	AT_{II}/8-Gly-AT_{II} pA_2	4-Phe-AT_{II}/8-Gly-AT_{II} pA_2	AT_{II}/8-Leu-AT_{II} pA_2
Rat stomach	6·90	6·90	8·16
Rat colon	7·40	7·42	
Rabbit aorta	7·42	7·35	8·21

Isolated organ bath (isometric recording)

Tissue	AT_{II}/8-Gly-AT_{II} pA_2 5′	AT_{II}/8-Gly-AT_{II} pA_2 15′*	AT_{II}/8-Leu-AT_{II} pA_2 5′	AT_{II}/8-Leu-AT_{II} pA_2 15′*
Rat stomach	7·17 ±0·05 (8)	7·06 ±0·03 (7)	8·03 ±0·04 (6)	7·97 ±0·04 (6)
Rabbit aorta	7·14 ±0·03 (7)	7·20 ±0·05 (5)	7·95 ±0·10 (4)	8·21 ±0·03 (6)

*Time of contact of antagonist with the tissue.
The number of estimations of pA_2 is given in parentheses.

One example of the experimental protocol used to estimate pA_2 in the three tissues, suspended in a cascade superfusion system, is presented at the top of Figure 4. The dose response curves of AT_{II} and of 4-Phe-AT_{II} in the presence of 8-Gly-AT_{II} and 8-Leu-AT_{II} remain parallel over a considerable range of concentrations of antagonists, although a relatively high dose of 8-Gly-AT_{II} (5×10^{-7}M) tends to flatten the slope of the curve of 4-Phe-AT_{II} on the rat colon. The middle range of the response curve was used to plot the logarithm of the dose-ratio-1 against the negative logarithm of the concentration of the antagonist (Arunlakshana & Schild, 1959) as in Figure 4. pA_2 values were derived graphically and are summarized in the upper part of Table 5; they are similar for the three tissues. The slopes of the straight lines are very close to unity, with the exception of 4-Phe-AT_{II}/8-Gly-AT_{II} on the rat colon, where the slope is 1·5. These results indicate that 8-Gly-AT_{II} is competitive antagonist of AT_{II} and 4-Phe-AT_{II} on the rat stomach and on the rabbit aorta.

Values of pA_2 estimated according to Schild (1947) from experiments on rat stomach and rabbit aorta, suspended in an isolated organ bath, are reported in the second part of Table 5. These values are very similar to those obtained in the experiment above. Moreover, pA_2 values measured after 5 and 15 min of contact of the antagonist with the tissues do not differ significantly, thus indicating that equilibrium conditions have been obtained after 5 min both with 8-Gly-AT_{II} and 8-Leu-AT_{II}.

Discussion

Replacement of 8-Phe in AT_{II} with aliphatic amino acids results in compounds that are inactive on isolated organs sensitive to AT_{II}, have high affinity for the receptors of AT_{II} and prevent the smooth muscle stimulating action of AT_{II} (Regoli *et al.*, 1971; Regoli & Park, 1972). Some of these compounds (8-Gly-AT_{II} and 8-Leu-AT_{II}) have recently been characterized on the rat stomach strip as potent, competitive and specific inhibitors of AT_{II} (Rioux, Park & Regoli, 1973; Regoli,

Park & Rioux, 1973). The replacement of the other residues of AT_{II} (amino acids 1 to 7) gives compounds showing variable decrease of potency, but no antagonism (Regoli & Park, 1972). It appears that, in spite of the large number of potentially active groups in its side chains, angiotensin stimulates the receptors only through the phenyl ring of 8-Phe.

The present experiments with 4, 6 and 7-Ala-AT_{II} confirm previous observations by Regoli & Park (1972), indicating that 4-Tyr and 6-His are important for binding the angiotensin molecule to the receptors. The Ala derivatives are characterized by a marked loss of potency and by the absence of antagonistic effect; moreover, the maximum contraction elicited by these compounds is similar to that of AT_{II}. Analogues of AT_{II}, in which 4-Tyr and 6-His have been substituted with Gly or Acpc, lose potency, show no antagonism, but give maximum effects significantly lower than AT_{II}. The difference between the two groups of analogues may be due to the fact that the substitution of 4-Tyr and 6-His with Gly or Acpc probably changes the secondary structure of AT_{II}, while the substitution with Ala does not. The same can be said to explain the difference between 7-Ala-AT_{II} and the other two derivatives in which 7-Pro has been replaced with Gly and Acpc. However, 7-Ala-AT_{II} is much more potent than 4-Ala-AT_{II} and 6-Ala-AT_{II}, thus suggesting that 7-Pro plays a smaller role in the binding of AT_{II} to receptors, than 4-Tyr and 6-His.

Compounds producing full size maximum responses were chosen for comparison of the receptors of AT_{II} in different tissues. The experiments showed that the order of potency of the 4 angiotensin derivatives in the three tissues is the same; this constitutes the first criterion for suggesting the identity of receptors for AT_{II} in the three preparations.

The action of the same compounds is abolished by desensitizing the tissues with AT_{II}, though the effects of 5-hydroxytryptamine, acetylcholine and noradrenaline are unchanged. These results have been used as a second criterion to suggest the identity of AT_{II} receptors in vascular and intestinal smooth muscle.

The third criterion (identity of pA_2 values for two antagonists in the three preparations) has also shown no clear differences between receptors for AT_{II} in the three tissues. pA_2 values for rat stomach strip and rabbit aorta have been measured by two different techniques: the cascade superfusion of Vane (1964) and the conventional isolated organ bath. In the first instance, pA_2 values have been estimated with the method of Arunlakshana & Schild (1959) and in the second, by the method of Schild (1947) (see Table 5). The results of this comparison show that the differences in pA_2 values, obtained with the two methods, do not exceed 0·3; moreover, the results of these experiments indicate that the cascade superfusion technique can be used satisfactorily for this kind of study.

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APPENDIX

Synthesis of angiotensin II (AT_{II}) analogues by solid-phase method

The following new peptides were synthesized according to the method of Merrifield (1963).

1	2	3	4	5	6	7	8		
Asp	Arg	Val	Phe	Ile	His	Pro	Phe	(4-Phe,5-Ile)-angiotensin II	(I)
,,	,,	,,	Gly	,,	,,	,,	,,	(4-Gly,5-Ile)-angiotensin II	(II)
,,	,,	,,	Tyr	,,	Gly	,,	,,	(5-Ile,6-Gly)-angiotensin II	(III)
,,	,,	,,	,,	,,	His	Gly	,,	(5-Ile,7-Gly)-angiotensin II	(IV)

The synthesis of octapeptides was carried out in a stepwise manner as described by Park *et al.* (1967). The Boc-phenylalanine (C-terminal amino acid) of the future peptide was esterified to chloromethylated polystyrene. The complex was then

introduced into the reaction vessel (Park & Regoli, 1972) in which all steps of the synthesis were carried out. The cycle for each amino acid consisted of removing the Boc group with 1·2 N HCl in acetic acid (AcOH), neutralization of the resulting hydrochloride with triethylamine (Et_3N) in *N,N'*-dimethylformamide (DMF) and then coupling the free base with the next amino acid using *N,N'*-dicyclohexylcarbodiimide (DCCI) as condensing agent. Solvents were redistilled at individual boiling points for purification. DMF was treated with P_2O_5 for one hour before distillation. At the end of all syntheses, the protected peptides were cleaved from the polymer by bubbling HBr through a suspension of peptide polymer in trifluoroacetic acid (TFA) and the partially protected peptides were catalytically hydrogenated to free peptides. All crude free octapeptides were purified by chromatography on a column of Sephadex G-25 using suitable solvents, and peptides were shown to be homogeneous by paper chromatography, thin-layer chromatography, electrophoresis, elemental analysis and amino acid analysis after acid hydrolysis.

Materials

All amino acids used were the L-isomer except glycine. *t*-Butoxycarbonyl (Boc)-amino acids, nitro-arginine, valine, isoleucine, proline, phenylalanine and glycine, were synthesized according to the procedure of Schwyzer, Sieber & Kappeler (1959). Boc-amino acid derivatives, *β*-benzyl-aspartic acid, *O*-benzyl-tyrosine, and imidazol-benzyl-histidine were obtained from Mann Research Lab., New York. Purity of amino acid derivatives and peptides was determined by paper chromatography on Whatman, No. 1, paper and by thin layer chromatography (Eastman Kodak 6060 silica gel sheets). The following solvents in an ascending system were used for both paper and thin layer chromatography: (1) 1-butanol:acetic acid:water (BAW) (4:1:5), and (2) 1-butanol:acetic acid:pyridine:water (PAPW) (30:6:20:24). Electrophoresis was carried out on S&S 2043A filter paper strips at 450 V, with formic acid–acetic acid buffer (pH 1·95) for 3 h at room temperature. Migrations are indicated by the ratio of the distance between the peptide and L-glutamic acid and abbreviated as E_G. Melting points (m.p.) were taken on a Kofler hot stage apparatus and are uncorrected.

For amino acid analysis the samples were hydrolyzed in 6 N HCl in sealed tubes (under nitrogen) at 110° C for 40 h and analysed on a Technicon amino acid auto-analyser. Micro analyses are indicated only by symbols of the elements and analytical results obtained for those elements were within ±0·4% the theoretical values.

Synthesis of peptides

Boc-phenylalanine polymer

Boc-phenylalanine (20 mmol) and Et_3N (21 mmol) in ethanol (EtOH)—ethyl-acetate (EtOAc) (1:1) mixture were refluxed with stirring for 24 h with 20 g of chloromethylated copolystyrene—2%—divinylbenzene and then for another 24 h Et_3N (5 mmol) was added to the reaction mixture and shaken at room temperature. The esterified polymer was removed by filtration, washed with EtOH—EtOAc (1:1); then, with EtOH, water and methanol (MeOH) separately and dried over NaOH, paraffin and P_2O_5 under vacuum. Each gram of polymer was found to contain 0·6 mmol of the Boc-phenylalanine.

Boc-β-benzyl-Aspartyl-nitro-Arginyl-Valyl-Phenylalanyl-Isoleucyl-imidazol-benzyl-Histidyl-Prolyl-Phenylalanine Polymer

Five grams (3·0 mmol of Boc-phenylalanine) of Boc-phenylalanine-polymer was introduced into the reaction vessel and the following steps were used to introduce each new amino acid residue. (1) Washing with glacial AcOH (3×60 ml). (2) Removal of Boc group by treatment with 50 ml of approx. 1·2 N HCl in AcOH for 30 minutes. (3) Washing with glacial AcOH (3×60 ml). (4) Washing with absolute EtOH (3×60 ml). (5) Washing with DMF (3×60 ml). (6) Neutralization of the HCl salt with 10 ml of Et_3N in 60 ml of DMF for 10 minutes. (7) Washing with DMF (3×60 ml). (8)* Washing with methylene chloride (CH_2Cl_2) (3×60 ml). (9) Addition of 6 mmoles of the appropriate Boc-amino acid dissolved in 50 ml of CH_2Cl_2 and mixing for 10 minutes. (10) Addition of 6·2 mmoles DCCI dissolved in 15 ml of CH_2Cl_2 and shaking the mixture for 3 h at room temperature. (11) Washing with CH_2Cl_2 (3×60 ml). (12)* Washing with absolute EtOH (3×60 ml). For coupling the amino acids Boc-imidazol-benzyl-histidine and Boc-nitro-arginine, step 8 was deleted and DMF was used as solvent in place of CH_2Cl_2 in steps 9 to 11.

* At this point, a small sample was put into a glass tube (i.d.=5 mm, *l*=50 mm), and 5 drops of ninhydrin were added. Then, the sample was heated at 80–90° C for 3–5 min for ninhydrin colour test.

Aspartyl-Arginyl-Valyl-Phenylalanyl-Isoleucyl-Histidyl-Prolyl-Phenylalanine (4-Phe,5-Ile)-angiotensin II . . . (I)

The protected peptide polymer was suspended in about 100 ml of anhydrous TFA and a gas dispersion tube was inserted into the side-arm of reaction vessel (Park & Regoli, 1972). Dry HBr was bubbled through the suspension by the gas dispersion tube for 45 minutes. The polymer was removed by filtration and washed three times with 20 ml portions of TFA. The combined filtrates were evaporated on a rotary evaporator under vacuum at 20° C and the product obtained was triturated with anhydrous ether. The amorphous powder obtained was collected on a sintered glass funnel (porosity F), washed well with anhydrous ether, dissolved in 60 ml of MeOH—AcOH—water (5:2:2), and reduced by bubbling H_2 through the solution at atmospheric pressure for 48 h with Pd-black (1·5 g for the first 24 h and 0·7 g for another 24 h: total 2·2 g) as catalyst.

The crude free octapeptide was purified by chromatography on a column of Sephadex G-25 coarse (85×5·0 cm) with BAW as developing solvent. Fractions of 8 ml each were collected with an automatic fraction collector. The homogeneity of the peptide was determined by ultraviolet (u.v.) absorption, paper and

TABLE 6. *Physical constants and yield of angiotensin II analogues*

Peptides	Paper chromatography (R_F) BAW	BAPW	Thin layer chromatography (R_F) BAW	BAPW	EG	m.p. °C	Yield (%)
(4-Phe,5-Ile)-AT_{II} (I)	0·58	0·51	0·45	0·70	1·21	210~213	55
(4-Gly-,5-Ile)-AT_{II} (II)	0·36	0·21	0·37	0·72	1·25	198~211	60
(5-Ile,6-Gly)-AT_{II} (III)	0·52	0·41	0·53	0·76	0·99	211~214	53
(5-Ile,7-Gly)-AT_{II} (IV)	0·39	0·20	0·43	0·73	1·21	223~226	57

thin layer chromatography in various solvent systems, and paper electrophoresis. The single homogeneous spots were identified either with ninhydrin, diazotized sulphanilic acid or Sakaguchi's reagent. Physical constants and yield are given in Table 6. Amino acid ratios found: Asp, 1·02; Arg, 1·05; Val, 1·00; Ile, 1·00; His, 0·98; Pro, 0·96; Phe, 2·05. Anal. C_{50} H_{71} N_{13} O_{11}·CH_3COOH (m.w. 1066·20), C, H, N.

The same procedures were used to synthesize and analyse the following three peptides:

Aspartyl-Arginyl-Valyl-Glycyl-Isoleucyl-Histidyl-Prolyl-Phenylalanine (4-Gly,5-Ile)-angiotensin II . . . (II)

For physical constants and yield, see Table 6. Amino acid ratios on an acid hydrolysate: Asp, 1·01; Arg, 1·03; Val, 0·98; Gly, 1·00; Ile, 1·00; His, 1·02; Pro, 0·97; Phe, 1·04. Anal. C_{43} H_{65} N_{13} O_{11}·CH_3COOH (m.w. 1002·14), C, H, N.

Aspartyl-Arginyl-Valyl-Tyrosyl-Isoleucyl-Glycyl-Prolyl-Phenylalanine (5-Ile,6-Gly)-angiotensin II . . . (III)

For physical constants and yield, see Table 6. Amino acid ratios on an acid hydrolysate: Asp, 1·02; Arg, 1·01; Val, 1·03; Tyr, 0·94; Ile, 0·98; Gly, 1·00; Pro, 0·96; Phe, 1·00. Anal. C_{46} H_{67} N_{11} O_{12}·$2H_2O$ (m.w. 1002·12), C, H, N.

Aspartyl-Arginyl-Valyl-Tyrosyl-Isoleucyl-Histidyl-Glycyl-Phenylalanine (5-Ile,7-Gly)-angiotensin II . . . (IV)

For physical constants and yield, see Table 6. Amino acid ratios on an acid hydrolysate: Asp, 1·01; Arg, 1·03; Val, 1·00; Tyr, 0·96; Ile, 1·00; His, 0·98; Gly, 1·02; Phe, 1·02. Anal. C_{49} H_{67} N_{13} O_{12}·CH_3COOH (m.w. 1066·18), C, H, N.

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