Table II; amino acid ratios on an acid hydrolysate: Asp, 0.96; Arg, 0.98; Val, 1.00; Tyr, 0.92; Ile, 0.95; His, 1.02; Pro, 1.05; Phe, 1.00; on an enzymatic hydrolysate: Asp, 1.00; Arg, 1.01; Val, 1.08; Ile, 0.98; His, 0.51; Pro, 0.58; (OMe)Tyr, 1.07; Phe, 1.00. *Anal.* ($C_{51}H_{73}N_{13}O_{12}\cdot CH_3COOH$) C, H, N.

Aspartylarginylvalyl-*O*-methyltyrosylvalylhistidylprolylphenylalanine ([4-(OMe)Tyr,5-Val]-angiotensin II).—The free octapeptide was prepared as described above to give 56% yield of the desired peptide; see Table II; amino acid ratios on an acid hydrolysate: Asp, 0.92; Arg, 1.06; Val, 2.13; Tyr, 0.98; His, 1.08; Pro, 1.00; Phe, 1.00; on an enzymatic hydrolysate, Asp, 1.00; Arg, 1.01; Val, 2.12; His, 0.58; Pro, 0.61; Phe, 1.00; (OMe)Tyr, 1.00. *Anal.* ($C_{50}H_{71}N_{13}O_{12}\cdot CH_3COOH$) C, H, N.

Asparaginylarginylvalyl-*O*-methyltyrosylvalylhistidylprolylphenylalanine ([1-Asp(NH_2),4-(OMe)Tyr,5-Val]-angiotensin II).—Woodward's Reagent K[24] (0.633 g, 2.5 mmol) was dissolved in 25 ml of DMF with vigorous stirring. At 0°, 0.67 g (2.5 mmol) of carbobenzoxyasparagine and 0.35 ml (2.5 mmol) of Et_3N dissolved in 25 ml of DMF were added. Stirring was continued until the solu cleared (about 3 hr). This was then added to a suspension of 2.5 g of heptapeptide polymer (nitroarginylvalyl-*O*-methyltyrosylvalyl-N^{im}-benzylhistidylprolylphenylalanine polymer) suspended in 25 ml of DMF containing 0.35 ml (2.5 mmol) of Et_3N. This heptapeptide polymer was prepared as described above. The reaction mixture was shaken in an ice bath for 2 hr, then at room temperature overnight. The resulting protected peptide polymer was collected by filtration, washed [DMF (3 times with 50 ml), absolute EtOH (3 times with 50 ml)], and finally dried over P_2O_5 *in vacuo* to yield 2.53 g.

The protected peptide polymer was suspended in 50 ml of anhydrous F_3CCO_2H and a slow stream of HBr was passed through with occasional shaking for 30 min under anhydrous conditions. The suspension was filtered and the polymer was washed (3 times with 8 ml) with anhydrous F_3CCO_2H. The combined filtrate was concentrated *in vacuo* at 20° and peptide was precipitated by addition of anhydrous Et_2O. The solid was removed by filtration and washed with anhydrous Et_2O. This partially protected octapeptide was dissolved in MeOH–AcOH–H_2O (10:1:1) and hydrogenated over Pd black for 48 hr. The peptide was isolated in the usual manner to yield 529 mg of solid. This was purified by chromatography on a Sephadex G-25 (coarse) column by elution with BAW solvent. The peptide emerged mainly as one fraction, was precipitated from 50% AcOH with Et_2O–Me_2CO (1:1), and dried over P_2O_5, NaOH, and paraffin *in vacuo* to yield 490 mg (50% yield based on 0.84 mmol of N-terminal nitroarginyl heptapeptide with polymer); see Table II; ratios on an enzymatic hydrolysate: Asp(NH_2), 0.95; Arg, 1.05; Val, 1.92; His, 0.51; Pro, 0.58; Phe, 1.00; (OMe)Tyr, 0.98. *Anal.* $C_{50}H_{72}N_{14}O_{11}\cdot 2CH_3COOH\cdot 2H_2O$ (1200.67): C, 53.97; H, 7.05; N, 16.33. Found: C, 53.42; H, 6.50; N, 16.09.

(24) (a) R. B. Woodward and R. A. Olofson, *J. Amer. Chem. Soc.*, **83**, 1007 (1961). (b) R. B. Woodward, R. A. Olofson and H. Mayer, *ibid.*, 1010 (1961).

Analogs of Angiotensin II. II. Mechanism of Receptor Interaction[1]

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[5-Ile]-angiotensin II has two sites of action on guinea pig ileum. It directly interacts with receptor sites on smooth muscle, leading to contraction, and also indirectly contracts muscle by interacting with receptor sites on the parasympathetic innervation of the ileum, releasing acetylcholine. An attempt has been made to study these two receptors, using responsiveness of the tissue to analogs of angiotensin II. Substitutions in positions 1–7 showed close correlations between pressor activity, smooth muscle activity, and release of acetylcholine. Substitutions in position 8, however, indicated that [5-Ile,8-(OMe)Tyr]-angiotensin II is about three times more active on guinea pig ileum than on blood pressure, while [5-Ile,8-Tyr]-angiotensin II has roughly the same activity in both. The release of acetylcholine by both peptides was the same; the smooth muscle response to the latter peptide was the same as the parent compound, while response to the former peptide was less than half that of the parent compound. On the other hand [5-Ile,8-Ala]-angiotensin II produced no response on guinea pig ileum similar to its effect on blood pressure, but it inhibited subsequent response to the parent compound. It is assumed that the analog binds to receptor sites producing no excitation but preventing other analogs from interacting. These results have been interpreted by a speculative scheme concerning conformations of muscle and nerve receptors.

The octapeptide, angiotensin II, is known to have a multiplicity of actions.[2] Until recently, angiotensin II analogs were usually assayed either by their pressor responses in ganglion-blocked or nephrectomized rats, or by their musculotropic effects on isolated rat uteri. In general, biological activity in these assay systems was roughly equivalent and this led to an assumption that angiotensin acted only on the smooth muscle cells in these two assay systems, and that the receptor sites on these cells were, at least grossly, similar.

Recently, Peach, Bumpus, and Khairallah[3] reported that angiotensin II inhibited uptake of norepinephrine into sympathetic nerve endings in rabbit heart, at dose levels of less than 50 pg/ml. Angiotensin II analogs substituted in positions 1–7 showed a close correlation between pressor/musculotropic activity and inhibition of uptake. Substitutions in position 8, however, indicated that the benzene ring of phenylalanine was not needed for inhibition of uptake but was required for pressor activity. Substituting phenylalanine by aminophenylbutyric acid or aminophenylisobutyric acid also dissociates inhibition of uptake from the pressor response. The free C-terminal CO_2H was required for both activities. This led to the conclusion that angiotensin receptor sites on sympathetic nerve endings were very similar to those on smooth muscle cells, except that the latter required an aromatic ring structure in position 8, while the former did not.

To study receptor sites on other tissues, guinea pig terminal ileum was used. Response of ileum to angiotensin is biphasic, a rapid component due to release of ACh from the intrinsic parasympathetic nerve ganglia of Meissner and Auerbach or postganglionic nerve end-

(1) This investigation was supported by U. S. Public Health Service Research Grant HE-6835 from the National Heart Institute.

(2) I. H. Page and J. W. McCubbin, Ed., "Renal Hypertension," Year Book Medical Publishers, Inc., Chicago, Ill., Chap. 5 and 9 (1968).

(3) M. J. Peach, F. M. Bumpus, and P. A. Khairallah, *J. Pharmacol. Exp. Ther.*, **167**, 291 (1969).

ings, and a slower component due to a direct interaction of angiotensin with smooth muscle cells.[4-6] The rapid response was blocked by atropine and morphine and potentiated by physostigmine[3] while the slower response was inhibited by 1-[4,4-bis(4-fluorophenyl)-butyl]-4-[2,6-dimethylanilinocarbonylmethyl]piperazine (lidoflazine).[6] By analyzing the patterns of response of the guinea pig ileum to angiotensin and its analogs we have tried to gain some information about angiotensin receptors in different tissues.

Methods

Female guinea pigs weighing about 500 g were killed by decapitation. A 3–4 cm piece of terminal ileum next to the ileocecal junction was cleaned and mounted as described previously.[3] Both isotonic contractions[3] and isometric responses measured by a force displacement transducer (Grass) were recorded. Dosages of peptides added are given as the final concentration per ml in the muscle bath. This dose was allowed to act on the ileum for 5 min and responses were measured. The muscle was washed a minimum of three times and a maximum of ten times with physiological salt solution and allowed to recover for a further 10 min before the next peptide was added. Drugs that modified responses to angiotensin were added 3–5 min before the peptide. All peptides were synthesized in our laboratory.[7]

Results

Overall biological activity of angiotensin analogs, recorded as isotonic contractions, are listed in Table I.

TABLE I

PRESSOR RESPONSE COMPARED TO ISOTONIC CONTRACTILE RESPONSE OF ISOLATED GUINEA PIG ILEUM

	Isotonic contractile response, %	Pressor response, %
[1-Asp(NH_2),5-Ile]-Angiotensin II	100.0	100.0
[5-Ile]-Angiotensin II	100.0	100.0
[5-Ile,4-(OMe)Tyr]-Angiotensin II	0.2	1.0
[1-Ile,5-Ile]-Angiotensin II	20–25	20.0
[5-Ile,8-(OMe)]-Angiotensin II	80–100	33.0
[4-Ala,5-Ile]-Angiotensin II	0.2	0
[5-Ile,6-Ala]-Angiotensin II	0.1	0.8
[5-Ile,7-Ala]-Angiotensin II	0.2	0
[5-Ile,8-Tyr]-Angiotensin II	60.0	83.0
[5-Ile]-Angiotensin I	10–15	
[5-Ile,8-Ala]-Angiotensin II	0.1	0.1

In general, it can be seen that pressor and contractile responses were roughly parallel with the exception of [5-Ile,8-(OMe)Tyr]-angiotensin II.

With isometric contractions, there also was a rough parallelism between pressor responses and both the direct musculotropic and indirect acetylcholine mediated responses. The major exception was observed when using peptides substituted in position 8. When [5-Ile,8-Ala]-angiotensin II was given in doses over 500 ng/ml, no contractile response was elicited. Two to three minutes later, [1-Asp(NH_2),5-Ile]-angiotensin II at 4 ng/ml did not elicit a response even though [5-Ile,8-Ala]-angiotensin II was washed out 1–3 times (Figure 1). Following repeated washings and a wait of 10–15 min, responses to [1-Asp(NH_2),5-Ile]-angiotensin II returned. Addition of [5-Ile,8-Ala]-angiotensin II again blocked further response, although responses to serotonin and vasopressin were not blocked. [1-Ile,-5-Ile]-angiotensin II in doses over 100 ng/ml also blocked responses to further doses of the same analog or [1-Asp(NH_2),5-Ile]-angiotensin II. This blockade lasted a long time even after 4–5 washings. Responses usually returned after 0.5-hr wait. None of the other analogs tested exhibited tachyphylaxis in the doses used.

Replacing phenylalanine in position 8 with tyrosine changed the pattern of isometric response. The rapid initial contractile phase was the same as with [1-Asp(NH_2),5-Ile]- or [1-Asp,5-Ile]-angiotensin II, but the second musculotropic phase was reduced by roughly 60% (Figure 2), as best seen when the first component is blocked with atropine (Figure 3). Thus, the ratio between the initial fast response and the slower muscle response is much greater with [5-Ile,8-Tyr]-angiotensin II than with the parent compound. Blocking the hydroxyl group as in [5-Ile,8-(OMe)Tyr]-angiotensin II changed the response again, making it similar to [1-Asp(NH_2),5-Ile]-angiotensin II. This difference is brought out even more when an acetyl cholinesterase inhibitor is added to the muscle bath (Figure 4). This enhances the first response without changing the muscle component.

Discussion

Smooth muscle contractile response can be recorded in two ways: isotonically and isometrically. With intestinal smooth muscle isotonic contractions usually are recorded with a simple lever and measure an overall shortening of the muscle strip. Since the muscle changes shape, transient alterations in tension are not recorded. Thus, isotonic contractions can be used when dose–response curves are needed, or when one wants to study structure–activity relationship. Isometric contractions, on the other hand, record transient tonic changes with only minimal motion. Thus, it can be used to analyze mechanisms of contractile responses, and in intestinal strips can indicate whether response is a rapid one due to release of ACH, or a low one due to direct smooth muscle stimulation.[8]

Decreasing responses to repeated injections of an agonist has been described as tachyphylaxis.[9] This phenomenon occurs following repeated administration of angiotensin II both *in vivo*[10] and *in vitro*.[11] It has been postulated that this tachyphylaxis is due to saturation of angiotensin II receptor sites on vascular smooth muscle cell membranes in an *in vitro* system.[10] This may play a role *in vivo*, but with other variable factors such as rate of delivery of peptide, blood flow,

(4) P. A. Khairallah and I. H. Page, *Amer. J. Physiol.*, **200**, 51 (1961).
(5) P. A. Robertson and D. Rubin, *Brit. J. Pharmacol.*, **19**, 5 (1962).
(6) T. Godfraind, A. Kaba, and P. Polster, *ibid.*, **28**, 93 (1966).
(7) N. C. Chaturvedi, W. K. Park, R. R. Smeby, and F. M. Bumpus, *J. Med. Chem.*, **13**, 177 (1970).
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(10) K. D. Bock and F. Gross, *Circulation Res.*, **9**, 1044 (1961).
(11) P. A. Khairallah, I. H. Page, F. M. Bumpus, and R. K. Turker, *ibid.*, **19**, 247 (1966).

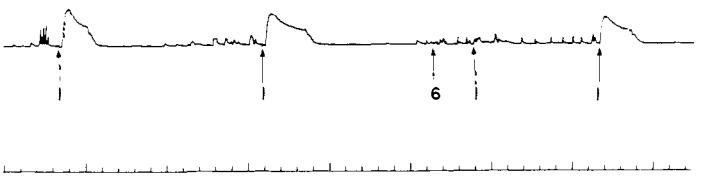

Figure 1.—Isometric contraction of guinea pig ileum. At 1, response to 4 ng/ml of [1-Asp(NH_2)]-angiotensin II and at 6, response to 500 ng/ml of [8-Ala]-angiotensin II. Between 6 and the succeeding 1, the ileum was washed twice. Time scale in minutes.

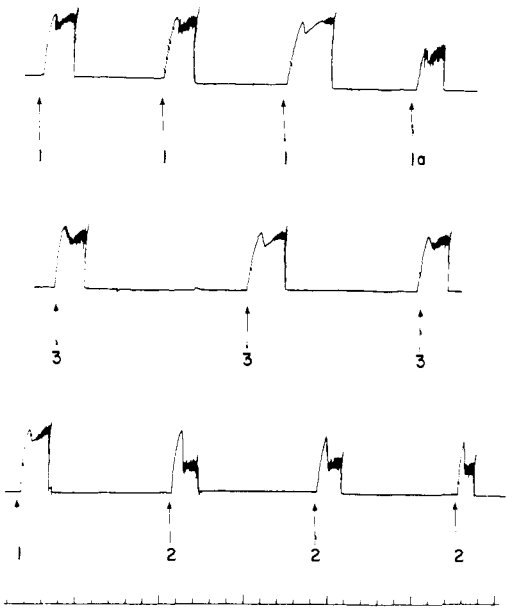

Figure 2.—Isometric contractions of guinea pig ileum. At 1, response to 4 ng/ml of [1-Asp(NH_2)]-angiotensin II; at 1a, response to 2 ng/ml of [1-Asp(NH_2)]-angiotensin II; at 2, response to 4 ng/ml of [8-Tyr]-angiotensin II; at 3, response to 4 ng/ml of [8-(OMe)Tyr]-angiotensin II.

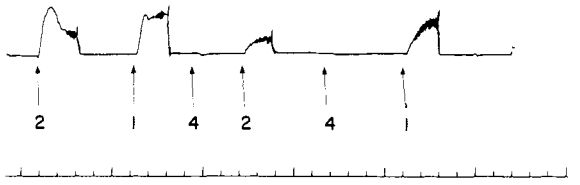

Figure 3.—Isometric contractions of guinea pig ileum. 1 and 2 are, respectively, 4 ng/ml of [1-Asp(NH_2)]-angiotensin II and [8-Tyr]-angiotensin II. At 4, 0.2 μg/ml of atropine was added to the bath followed by the peptide at 1 and 2.

$$\text{Ang} + \text{Rec} \underset{K_2}{\overset{K_1}{\rightleftarrows}} \text{AR} \xrightarrow{K_3} \text{degradation products}$$

Until the complex dissociated, no further reaction could occur; thus, tachyphylaxis resulted.

When dissociation of the complex occurs by enzymatic destruction (angiotensinase A) of the peptide, small fragments appear. Thus, K_3 is probably the main degradation pathway. K_2 is the reversal of K_1 which may not occur in nature, but does occur with the addition of resins like Dowex-50, that bind angiotensin stronger than the receptor.

With [1-Asp(NH_2),5-Ile]- or [1-Asp,5-Ile]-angiotensin II, K_1 is greater than K_2 or K_3, and when the peptide is given in large doses or at frequent intervals, an accumulation of complex (AR) occurs leading to tachyphylaxis. Of all the peptide analogs tested on isolated guinea pig ileum, only [5-Ile,8-Ala]- and [1-Ile,5-Ile]-angiotensin II produced cross-tachyphylaxis with angiotensin II; thus it must also interact with the same specific receptor site. Since very large doses of [5-Ile,8-Ala]-angiotensin II are required, the interaction between the analog and receptor site must be weak; therefore, more peptide is needed to drive the reaction to the right and form AR. Once the complex is formed, it is dissociated probably by enzymatic means through K_3. [1-Ile,5-Ile]-angiotensin II probably interacts with the receptor site in a manner similar to angiotensin II. However, since angiotensinase A is specific for N-terminal dicarboxylic amino acids,[12] this peptide is metabolized at a slower rate; thus K_3 is less, allowing more AR to accumulate.

One can conclude that in intestinal smooth muscle these two analogs and angiotensin II interact with the same receptor site, while most of the other analogs either do not, or the binding is so weak (very low K_1) that almost no complex is formed. Thus, for musculotropic action, the peptide must have a benzene or

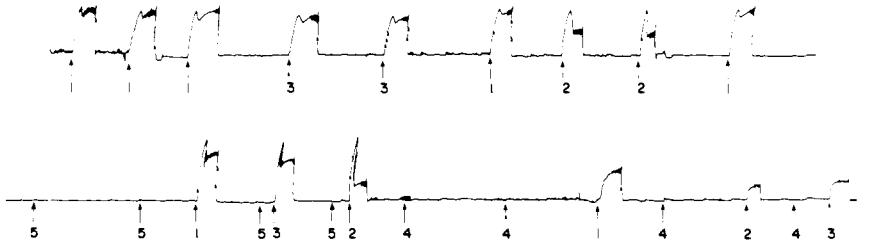

Figure 4.—Isometric contractions of guinea pig ileum. 1, 2, and 3 as in Figure 2. At 5, 0.02 μg/ml of physostigmine (a cholinesterase inhibitor) was given followed by the peptide. At 4, atropine (0.2 μg/ml) was given.

enzymes, etc., explanation of tachyphylaxis possibly is more complicated. An assumption was made that angiotensin II interacted with its receptor to form a complex (AR) (Figure 5) and the formation of the complex then led to a response.

phenyl group in position 8, and free C terminus to interact with receptor sites. Once the complex is formed, it is fairly stable and may be reversed by metabolism of the N terminus by such enzymes as angiotensinase A.

(12) P. A. Khairallah and I. H. Page, *Biochem. Med.*, **1**, 1 (1967).

In intestinal smooth muscle, angiotensin II has two sites of action, the smooth muscle and the parasympathetic chain. To cause the release of ACh the peptide molecule must interact either with the ganglion cell or the postganglionic nerve terminals. The specificity of these sites can be determined by studying the rapid component of intestinal response and compare this to the slow component. Most of the angiotensin analogs tested showed the same pattern of response as did the parent compound, with the exception of [5-Ile,8-Tyrl]-angiotensin II. The fast component was almost the same as that seen with [5-Ile]-angiotensin II, although the slow component was much less. This was demonstrated more clearly when the fast component was blocked by atropine. The slow component in this case was less than half that of the slow component of [5-Ile]-angiotensin II.

When the OH group of tyrosine 8 was methylated, the pattern resembled that of [5-Ile]-angiotensin II instead of [5-Ile,8-Tyr]-angiotensin II. Thus, one may conclude that receptor sites on the parasympathetic nerves cannot distinguish between [5-Ile,8-Phe]- and [5-Ile,8-Tyr]-angiotensin II while receptor sites in smooth muscle cells can. Since the smooth muscle response is the same to [5-Ile,8-(OMe)Tyr]-angiotensin II and to the parent compound while it is much less to the 8-tyrosine analog when comparable doses are used, the binding of the first two compounds to receptor sites can occur independently of steric effects of the methoxy group on the phenyl ring. [5-Ile,8-Tyr]-angiotensin II, however, can form an additional H bond with the phenolic OH and thus may bind to a site other than the specific receptor, but close to it (Figure 5).

On sympathetic nerve endings [5-Ile]-angiotensin II and certain of its analogs inhibit reuptake of norepinephrine.[2] Here again, changes in positions 1–7 of the peptide molecule showed a close correlation between pressor activity and inhibition of uptake and again changes in position 8 led to a separation of the two biological parameters. [5-Ile,8-Ala]-, [5-Ile,8-(3-amino-3′-phenyl)isobutyric acid]-, and [5-Ile,8-(3-amino-4-phenyl)butyric acid]-angiotensins II all inhibited uptake to approximately the same degree as the parent compound, while the pressor activity was very low. Thus, not only does [5-Ile,8-Ala]-angiotensin II bind to receptor sites on the sympathetic axonal varicosities, but it also produces a biological response. This must indicate that the sympathetic and parasympathetic receptor sites differ slightly.

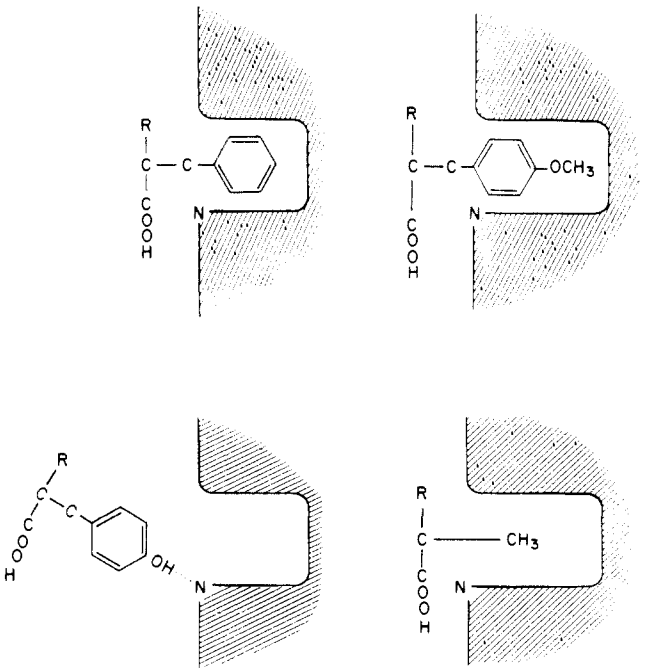

Figure 5.—Schematic representation of possible angiotensin receptor sites on intestinal smooth muscle cell membrane. The 8-position of angiotensin must interact with smooth muscle membrane receptor sites, and the interaction leads to a response. The same response can occur with [8-(OMe)Tyr]-angiotensin II, indicating that the large OCH_3 does little to change both the interaction with and response of the muscle. [8-Ala]-angiotensin II can cover the receptor sites preventing other molecules from interacting, but since it does not contain a benzene side chain, no response is obtained. Finally, with [8-Tyr]-angiotensin, although there should be no steric hindrance, there is no or only incomplete interaction with the receptor site, probably due to H-binding between the free hydroxyl of tyrosine and a H-bond acceptor adjacent to the receptor.