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EFFECT OF NERVE STIMULATION AND ANGIOTENSIN ON THE ACCUMULATION OF ^{3}H-NOREPINEPHRINE AND THE ENDOGENOUS NOREPINEPHRINE LEVEL IN GUINEA PIG VAS DEFERENS*

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Abstract—The effect of angiotensin on the endogenous norepinephrine (NE) content and on the accumulation of ^{3}H-NE was studied in the isolated guinea pig vas deferens stimulated at both the pre- and postganglionic level (hypogastric nerve and transmural stimulation). It was found that angiotensin blocked ^{3}H-NE accumulation (uptake and retention) and produced a depletion of endogenous NE in the unstimulated vas deferens. Continuous pre- and postganglionic stimulation at low-frequency (6/sec) did not produce any significant changes in the endogenous NE level, while high-frequency (50/sec) stimulation reduced it by 30–40 per cent. The accumulation of ^{3}H-NE was impaired by continuous pre- and postganglionic stimulation and was dependent on the frequency of stimulation. With a high-frequency (50/sec), the accumulation was only 5–6 per cent of the control, suggesting that depolarization of the neuronal membrane, produced by nerve stimulation, blocks the re-uptake of NE and favors its release. It appears, therefore, that these two phenomena are sequential for a certain area of the neuronal membrane. Angiotensin abolished the inhibiting effect of nerve stimulation on ^{3}H-NE uptake and retention. It is concluded that the effect of angiotensin on the endogenous NE level and on the accumulation of ^{3}H-NE depends on the degree of impulse activity in the sympathetic nerve and the concentration of angiotensin used. The duality (or plurality) of the action of angiotensin in the stimulated and unstimulated guinea pig vas deferens suggests a possible modulating role of angiotensin in adrenergic neurotransmission.

ANGIOTENSIN is a naturally occurring, physiologically highly active peptide that exhibits a central vasomotor effect[1] and influences autonomic cholinergic[2,3] as well as adrenergic neurotransmission.[4,5] We have shown that angiotensin inhibits the uptake of ^{3}H-norepinephrine (^{3}H-NE) in the spleen brain-stem slices, and aortic strips in the rat,[6] and in the perfused mesenteric bed of the cat.[7] This effect was due to the inhibition of the ^{3}H-NE uptake[8] and re-uptake at the level of the neuronalmembrane.

The present work deals with the hypothesis that, if angiotensin effect is due to the blockade of norepinephrine (NE) re-uptake at the neuronal membrane, then the membrane depolarization produced by nerve stimulation may possibly interfere with the action of angiotensin. In studying this problem, the isolated vas deferens was

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chosen because of its rich adrenergic innervation and the opportunity it affords for pre- and postganglionic stimulation.[10] Another reason for this choice was that angiotensin does not contract the vas deferens, an observation that suggests the absence of specific muscular receptors for angiotensin. The preparation is, therefore, especially suitable for the study of indirect, neurogenic effects of angiotensin.

Our results revealed that angiotensin decreases the level of endogenous NE and inhibits the accumulation of ^{3}H-NE in the unstimulated vas deferens. The accumulation of ^{3}H-NE is also inhibited by nerve stimulation, but this effect is reduced by angiotensin.

METHODS

Male guinea pigs weighing approximately 500 g were killed by decapitation; the vas deferens was removed and incubated in a 50-ml muscle bath containing modified Krebs solution[12] at 37°, for 1 hr. The vas deferens from one side served as the control for the contralateral organ which was treated according to the protocol. Electrical stimulation of the hypogastric nerve was performed as described by Hukovic,[11] and transmural stimulation according to the method of Birmingham and Wilson.[12] In both cases, monophasic square stimuli of supramaximal strength and 1-msec duration were applied continuously for 1 hr. Two different frequencies were chosen: 6 shocks/sec, which is in the range of normal physiological impulse flow in the sympathetic nerves,[13] and 50 shocks/sec. The contractions of the vas deferens were recorded on a smoked drum. For transmural stimulation, the vas deferens was dissected and cleaned of peritoneum and the surrounding fat tissue with the aid of a binocular dissecting microscope.

The accumulation of ^{3}H-NE was studied using the following procedure: Val^5-angiotensin amide (Hypertensin, Ciba) in concentrations of 1×10^{-8}M, 2×10^{-7}M, and 1×10^{-6}M, was added to the bath 10 min before ^{3}H-NE. The final concentration of ^{3}H-NE in the bath was 5 ng/ml (sp. act. 190 mc/mg, New England Nuclear Corp., Boston, Mass.). In order to prevent the oxidation of ^{3}H-NE, diaminoethane-tetra-acetic acid disodium salt (EDTA) (10 mg/l.) and ascorbic acid (20 mg/l.) were freshly prepared daily and added to the medium, which was constantly bubbled with 95% O_2 and 5% CO_2. The pH 7·4 was carefully controlled, since it was observed that even small changes in pH can profoundly change the ^{3}H-NE uptake. After 1 hr incubation, the preparations were washed twice in 5 cm^3 of saline for 30 sec, blotted dry on filter paper, and placed in a deep freezer.

Assay of total tissue NE. The tissue was homogenized in 5 ml of 0·1 N HCl and centrifuged. An aliquot of the clear supernatant was extracted and the total tissue NE was determined spectrofluorometrically (Aminco–Bowman spectrophotofluorometer) according to the method of Shore and Olin[14] as modified by Chang.[15] Since the amount of ^{3}H-NE taken up from the medium was very small (1–10 mg/g tissue) as compared with the content of endogenous NE (approx. 10 μg/g) in the vas deferens, it can be assumed that the NE determined by spectrofluorometry represented endogenous NE, rendering correction of these values by substracting the amount of ^{3}H-NE unnecessary.

Assay of ^{3}H-radioactivity. The ^{3}H-radioactivity was measured with a Packard liquid scintillation counter. ^{3}H-NE was separated on alumina columns and determined according to the method of Whitby *et al.*[16] An aliquot of the supernatant was passed

over the alumina columns and ^{3}H-NE was eluted with 0·2 N HCl; 1 ml of the eluate was then mixed with 10 ml of Bray's liquid[17] and counted. The values for radioactivity were corrected for the efficiency of counting. Statistical analysis was performed using the standard *t*-test (Student test).

RESULTS

Effect of continuous transmural and hypogastric nerve stimulation on the contractile response of the vas deferens. Continuous electrical stimulation at the postganglionic (transmural stimulation) and preganglionic levels (hypogastric nerve stimulation) for 1 hr, with monophasic square stimuli of supramaximal strength and 1-msec duration, resulted in diminution or complete cessation of the contractile responses of the vas deferens, depending on the frequency of stimulation. With 6/sec, the initial contraction persisted for 3–10 min, followed by relaxation of the vas deferens. After this initial period, a great number of short-lasting, irregular contractions were observed, but the height of these contractions never reached the level of the initial contraction (Fig. 1). With 50/sec, the initial contraction was followed by almost complete unresponsiveness or by very few, small, irregular contraction. There was no marked difference between the tracings obtained with hypogastric nerve stimulation and transmural stimulation. At a concentration of 2×10^{-7} M, angiotensin increased the frequency of contractions of the vas deferens stimulated at the pre- and postganglionic level in about 80 per cent of the assays; the tracings of a typical experiment are shown in Fig. 1. The contractile response of the vas deferens to angiotensin 10^{-8}M was not different from that of the control.

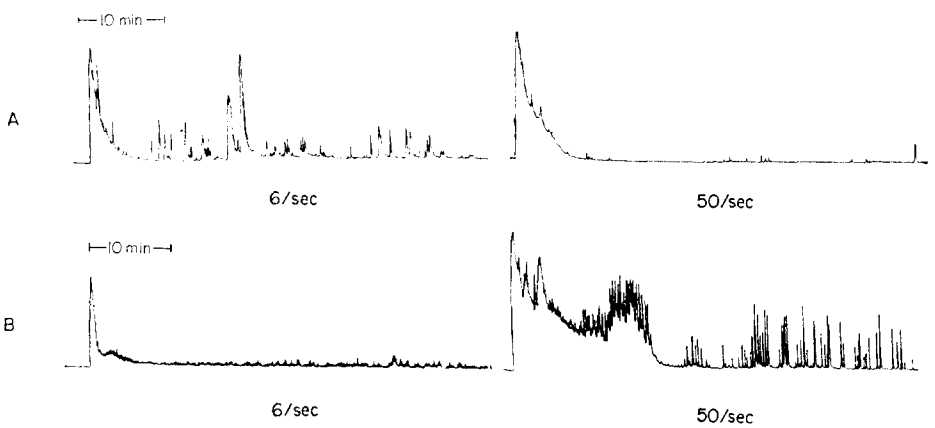

FIG. 1. Contractile responses of the vas deferens after continuous hypogastric nerve stimulation. A, Control. B, Angiotensin (2×10^{-7}M).

Effect of angiotensin on endogenous NE level in nonstimulated and stimulated vas deferens. As can be seen from the results presented in Table 1, the spontaneous output of endogenous NE during the 1-hr incubation period reduced the NE content by 50 per cent. Transmural and hypogastric nerve stimulation of the vas deferens with 6/sec did not further decrease endogenous NE. However, continuous stimulation at 50/sec significantly lowered the NE level.

In nonstimulated preparations, the spontaneous output of NE was inhibited by angiotensin at a concentration of 10^{-8}M and potentiated by 2×10^{-7}M and 10^{-6}M. The effect of angiotensin on endogenous NE in stimulated vas deferens preparations varied according to the type of stimulation and the concentration of angiotensin in the incubation medium. Angiotensin 10^{-8}M did not produce any additional changes in the NE content of the vas deferens stimulated transmurally or through the hypogastric nerve. On the other hand, angiotensin 2×10^{-7}M blocked the reduction of NE produced by stimulation of the hypogastric nerve or by transmural stimulation with 50/sec. Only in the case of low frequency transmural stimulation, i.e. postganglionic stimulation, was this concentration of angiotensin able to significantly potentiate ($P < 0.025$) the release of NE.

TABLE 1. EFFECT OF ANGIOTENSIN ON THE ENDOGENOUS NE (μg/g TISSUE) LEVEL IN STIMULATED AND NONSTIMULATED GUINEA PIG VAS DEFERENS*

			Angiotensin		
		Control	10^{-8}M	2×10^{-7}M	1×10^{-6}M
1-hr incubation	Nonincubated	10·71 ± 0·38			
	Nonstimulated	5·09 ± 0·26 < 0·001	7·80 ± 0·61 < 0·001	2·68 ± 0·40 < 0·001	4·00 ± 0·07 < 0·001
	Hypogastric nerve Stimulation 6/sec	5·41 ± 1·60 < 0·01	4·18 ± 0·38 n.s.	7·72 ± 0·74 n.s.	
	Hypogastric nerve Stimulation 50/sec	2·99 ± 0·72 < 0·001	4·11 ± 0·32 n.s.	8·06 ± 059 < 0·001	
	Transmural Stimulation 6/sec	5·46 ± 0·64 < 0·001	4·49 ± 0·13 n.s.	3·50 ± 0·40 < 0·025	
	Transmural Stimulation 50/sec	3·57 ± 0·09 < 0·001	3·59 ± 1·14 n.s.	8·40 ± 0·66 < 0·001	

*Mean of six experiments ± S.E.M. P values under control results express the level of significance between nonincubated control and appropriate incubated control. The results of the experiments with angiotensin were compared with corresponding control.

Effect of transmural and hypogastric nerve stimulation on the accumulation of ^{3}H-NE. The accumulation of ^{3}H-NE by the vas deferens was measured by incubation with 5 ng/ml of ^{3}H-NE for 1 hr. On the basis of recent work by Avakian and Gillespie,[18] the tracer dose of labelled NE (5 ng/ml) used in our experiments was low enough to ensure the accumulation of ^{3}H-NE exclusively by the nerve endings. In order to prevent oxidation of ^{3}H-NE, EDTA and ascorbic acid were added to the incubation medium. The amount of labelled NE taken up by the vas deferens and found after separation on alumina columns showed that more than 70 per cent of ^{3}H-radioactivity was present as ^{3}H-NE. Continuous preganglionic and postganglionic stimulation resulted in a reduction of accumulation of ^{3}H-NE, the extent of which varied with the frequency of stimulation. The amount of ^{3}H-NE found after transmural stimulation with 6/sec and 50/sec was decreased by 20 and 95 per cent, respectively, as compared with the nonstimulated control. Similar results were obtained with hypogastric nerve stimulation (Fig. 2).

Depending on the concentration used, angiotensin partially or completely abolished the inhibiting effect of nerve stimulation on ^{3}H-NE accumulation. Angiotensin

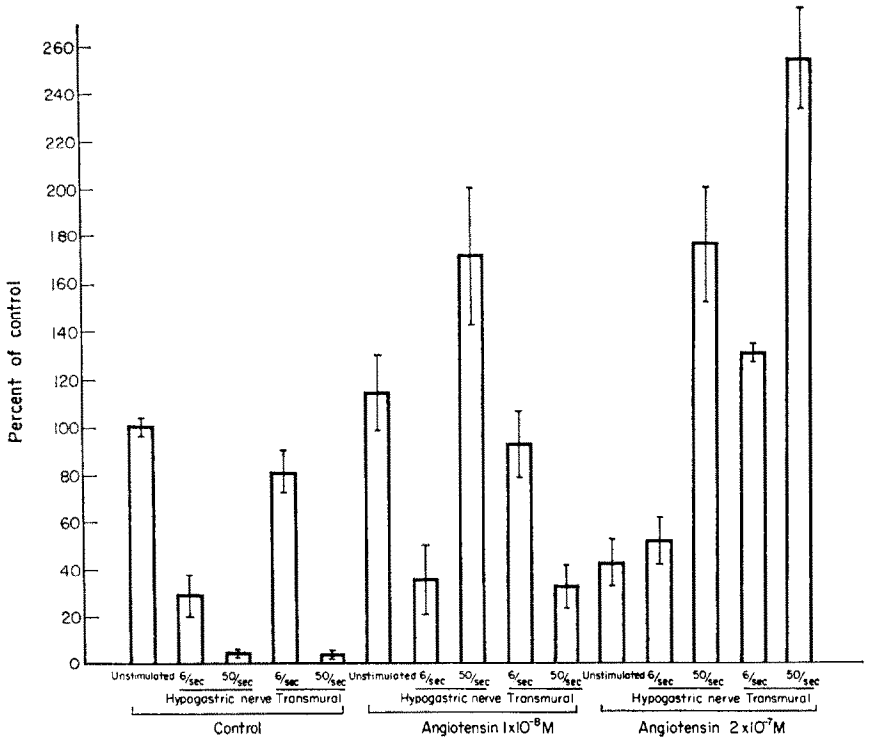

FIG. 2. Effect of continuous transmural or hypogastric nerve stimulation and angiotensin on the ^{3}H-NE accumulation by guinea pig vas deferens. Mean of six results ± S.E.M. The amount of ^{3}H-NE accumulated by unstimulated control was 231 ± 8 mμc per g tissue.

10^{-8}M abolished the effect of high-frequency stimulation only (50/sec), while angiotensin 2 × 10^{-7}M completely counteracted the effect of both low-and high-frequency stimulation on the accumulation of ^{3}H-NE. The content of ^{3}H-NE in the presence of angiotensin 2 × 10^{-7}M was even increased by 76 per cent ($P < 0.001$) when the hypogastric nerve was stimulated (50/sec) and by 155 per cent ($P < 0.001$) when the vas deferens was stimulated transmurally (50/sec).

DISCUSSION

Our previous investigations[8,9] showed that angiotensin affects the NE exchange (the uptake and release) at the level of the neuronal membrane. The present study was undertaken in order to assess more completely the effects of angiotensin on adrenergic neurotransmission in a smooth muscle on which it does not have a direct action. Measurement of the accumulation (uptake and retention) of labelled NE and determination of the endogenous NE level afforded the opportunity to study the changes at the presynaptic site, while the contractile responses to nerve stimulation served as an indicator of postsynaptic events.

The effect of angiotensin on the accumulation of ^{3}H-NE and the endogenous NE level varies according to the degree of excitation of the sympathetic nerve and the concentration of angiotensin used. The spontaneous output of endogenous NE is

stimulated by angiotensin at a concentration of 2×10^{-7}M or higher and, at the same time, is accompanied by reduced accumulation of ^{3}H-NE in unstimulated vas deferens preparations. This observation indicates that the depletion of NE caused by angiotensin is due to the increased release and reduced re-uptake and retention of NE. Libau *et al.*[19] observed a 40–50 per cent decrease of endogenous NE in rat aortic strips when a high concentration of angiotensin (1 mg/ml) was used. In our experiments, increasing the concentration of angiotensin from 2×10^{-7}M to 10^{-6}M did not further augment the NE depletion. It, therefore, appears that angiotensin affects only a certain portion of the NE stores ("angiotensin sensitive pool") and that the depleting effect is not proportional to the concentration of angiotensin used.

High-frequency (50/sec) hypogastric nerve stimulation and transmural stimulation reduced the endogenous NE content, while the low frequency (6/sec) was without effect. Similar results have been observed by other investigators.[20] When angiotensin was added to the incubation medium at a concentration of 2×10^{-7}M, which *per se* had a depleting effect on endogenous NE in nonstimulated preparations, it strongly inhibited the NE release produced by high-frequency nerve stimulation and thus prevented the excessive, unphysiological loss of NE. However, angiotensin (2×10^{-7} M) together with low-frequency postganglionic stimulation reduced the NE level, suggesting that under the low, physiological impulse flow in the sympathetic nerve, the action of angiotensin is directed toward an increased release of NE. This duality (or plurality) of action under different conditions of nerve stimulation indicates that angiotensin, as a local hormone, is a modulator of adrenergic neurotransmission. The results of the effect of angiotensin on the accumulation of ^{3}H-NE lend further support to this hypothesis.

Our findings show that the accumulation of ^{3}H-NE is impaired by continuous pre- and postganglionic stimulation, an effect that is frequency-dependent. Continuous stimulation of the hypogastric nerve with 50/sec inhibited the accumulation of ^{3}H-NE by 95 per cent while stimulation with 6/sec inhibited it by 70 per cent, transmural stimulation with 6/sec inhibited ^{3}H-NE accumulation only by 20 per cent and 50/sec inhibited it by 96 per cent. In explaining these results, it can be assumed that the depolarization of the neuronal membrane produced by nerve stimulation inhibits the re-uptake and favors the release of NE. Therefore, when the frequency of stimulation is high the depolarization would last longer and the inhibiting effect on the re-uptake becomes more evident. If this is true, then the release and the re-uptake are not simultaneous but sequential processes for a certain area of the synaptic membrane. This explanation can be supported by the following consideration: A 1-msec pulse applied fifty times per sec causes depolarization of the neuronal membrane for no longer than 250 msec,[21] while the inhibition of accumulation is almost complete. The only evidence to support the contention that nerve stimulation has an enhancing effect on the uptake of labelled NE was obtained in the perfused stimulated cat heart[22] and stimulated rat submaxillary gland:[23] intermittent or short-lasting stimulation of these organs produced an increased uptake of labelled NE. Recently, Malforms* obtained experimental evidence, using the tissue fluorescence technique, that supports our observation that continuous nerve stimulation prevents the uptake of NE. In the present experiments, low-frequency preganglionic stimulation produced a greater inhibiting effect on the accumulation of ^{3}H-NE than did low-frequency postganglionic stimulation,

*T. Malforms, personal communications.

suggesting that the synaptic relay in the ganglion multiplies the effect of preganglionic stimulation at the nerve terminals.

Angiotensin partially or completely abolished the inhibition of ^{3}H-NE accumulation caused by nerve stimulation, At a concentration of 10^{-8}M, inhibition was observed only when the vas deferens was stimulated with 50/sec, while it was observed for 6/sec and 50/sec with 2×10^{-7}M. Angiotensin even increased the accumulation of ^{3}H-NE by 76 per cent in the preparation stimulated preganglionically with 50/sec and by 155 per cent in the preparation stimulated transmurally (50/sec). These results are compatible with those on endogenous NE and can be interpreted similarly, on the basis of the assumption that angiotensin exerts a modulatory action at the adrenergic synapses. More specifically, when the release of NE produced by nerve stimulation becomes excessive, angiotensin counteracts this process and increases the accumulation (the uptake and retention) of NE by nerve terminals. However, when the release of NE is within a physiological range, angiotensin stimulates the releasing process.

The contractile responses observed after continuous pre- and post-ganglionic stimulation are not readily explainable in terms of presynaptic changes, i.e. the changes in endogenous NE content and the accumulation of NE, thus indicating once again that pre- and postsynaptic events are to some extent independent processes. This is understandable, since additional factors are involved, namely, the receptors, the diffusion barriers, the excitation–contraction mechanism in smooth muscle, etc. The cessation of contractile responses after high-frequency stimulation can be explained by recent theories on receptor mechanisms,[24] according to which an abundance of the released transmitter reaches the receptor sites, producing the desensitization ("saturation") of receptors. Low-frequency continuous nerve stimulation probably has the same effect but to the lesser extent and, therefore, the contractions are smaller and their incidence is determined by the availability of the free receptors at the smooth-muscle membrane. That angiotensin increases the number of contractions during continuous nerve stimulation possibly reflects the changes at the presynaptic site, i.e. the modulation of release and re-uptake of transmitter, as described above.

Finally, since there are some smooth muscles, like the vas deferens, on which angiotensin does not act directly but modulates the effect of nerve stimulation,[25] we should like to propose two types of angiotensin receptors: muscular and neural. This hypothesis is supported by studies of the structure–activity relationship of certain angiotensin analogues.* Activation of the muscular receptors at the membrane of the smooth muscle cell would be responsible for the direct action of angiotensin. The neural angiotensin receptors would participate in the process of transmitter exchange (release and re-uptake) at the neuronal membrane, through an ionic mechanism of unknown nature.

*P. A. Khailrallah, personal communication.

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