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Evidence that angiotensin enhances transmitter release during sympathetic nerve stimulation

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Summary

1. The effects of angiotensin on the contractility of the transmurally stimulated rabbit portal vein and coeliac artery have been studied in conjunction with its effects on the release and uptake of (±)-^{3}H-noradrenaline.

2. Angiotensin contracted both vein and artery; these responses were enhanced by veratrine and reduced by tetradotoxin. At low (non-contractile) concentrations of angiotensin, contractions elicited by electrical stimulation (0·5–4 Hz) had a quicker onset and reached a higher maximal tension than control responses. Higher concentrations of angiotensin increased the degree of potentiation. Contractions to noradrenaline were not enhanced by angiotensin.

3. Pretreatment of the coeliac artery, portal vein and perfused rat heart with angiotensin did not reduce the subsequent uptake of labelled noradrenaline in the presence of angiotensin. Simultaneous treatment with angiotensin and ^{3}H-noradrenaline caused a small, apparent inhibition of uptake into the portal vein.

4. When the portal vein was incubated with ^{3}H-noradrenaline there was a marked accumulation of label within the tissue; over 90% of the radioactivity retained in the tissue was identified as intact noradrenaline. When the vein, or artery, was superfused with amine-free Krebs there was a steady basal release of label; the greater proportion of this label was identified as deaminated metabolites. Electrical stimulation evoked a frequency dependent release of ^{3}H above basal levels. The greater proportion of this increased efflux was due to the release of intact ^{3}H-noradrenaline, with smaller increases in the amount of O-methylated and deaminated metabolites.

5. Angiotensin increased the efflux of labelled noradrenaline + normetanephrine, or of total ^{3}H, during transmural stimulation (0·5–4 Hz) in both the vein and artery, but did not increase the efflux of deaminated products during electrical stimulation. The output of labelled noradrenaline + normetanephrine was usually doubled in the presence of angiotensin (200–500 ng/ml) during electrical stimulation of the portal vein.

6. Cocaine (4 μg/ml) potentiated responses to noradrenaline and transmural stimulation, and doubled the output of ^{3}H or labelled noradrenaline + nor-

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metanephrine during electrical stimulation. Cocaine did not alter the potentiating effects of angiotensin.

7. Angiotensin elicited transitory increases in the basal efflux of ^{3}H from both the portal vein and coeliac artery. However, this did not account for the marked increase in efflux seen during electrical stimulation. Vasopressin did not potentiate responses to transmural stimulation or significantly influence the efflux of ^{3}H from the vein or artery.

8. Noradrenaline and 5-hydroxytryptamine, but not acetylcholine, markedly increased the efflux of ^{3}H from the portal vein. This increase in efflux of label is believed to result from an exchange of exogenous noradrenaline and 5-hydroxytryptamine with labelled tissue stores of noradrenaline.

9. It is suggested that angiotensin is able to facilitate the release of the sympathetic transmitter and that this is the basis for its action in potentiating the responses to sympathetic stimulation. It is also suggested that the noradrenaline releasing action of angiotensin (by excitation of sympathetic nerve endings) has led to confusion in previous investigations of the effects of angiotensin on noradrenaline uptake.

Introduction

Angiotensin has been shown to enhance responses elicited by post-ganglionic sympathetic nerve stimulation *in vivo* and *in vitro*, but there is considerable disagreement about the mechanisms of this potentiation. It has been suggested that angiotensin can increase the amount of noradrenaline (NA) released during nerve stimulation (Benelli, Della Bella & Gandini, 1964 ; Zimmerman & Gomez, 1965 ; Zimmerman & Gisslen, 1968 ; Day & Owen, 1969). However, others have presented evidence that the polypeptide acts by preventing the re-uptake of NA into sympathetic nerves (Palaić & Khairallah, 1967 ; Peach, Bumpus & Khairallah, 1969). Starke, Werner & Schumann (1969) have shown that angiotensin does increase NA release by sympathetic stimulation in the rabbit heart, but these results are consistent with either of the above hypotheses.

We decided to study both the uptake and release of ($\pm$)-^{3}H-NA in the same preparation in an attempt to resolve this problem. The longitudinal muscle of the rabbit portal vein provides an excellent test object for such a study since it is a robust tissue well endowed with a post-ganglionic sympathetic innervation (Hughes & Vane, 1967) and yet is thin enough to allow rapid transmitter diffusion into the perfusion media. A parallel study has also been carried out on the helical muscle preparation of the rabbit coeliac artery and these results are compared with those obtained in the vein. The effect of angiotensin on the uptake of ($\pm$)-^{3}H-NA in the two vascular tissues was also compared with its effect on uptake of label in the isolated, perfused, rat heart.

We shall present evidence that the potentiating effects of angiotensin can be explained by a facilitation of NA release during sympathetic nerve stimulation. A preliminary report of this work has been made to the British Pharmacological Society (Hughes & Roth, 1969).

Methods

The longitudinal muscle of the rabbit portal vein was dissected and prepared for transmural stimulation as described by Hughes & Vane (1967). For superfusion,

threads were attached to each end of the vein ; the upper thread was tied to an isometric force displacement transducer (Grass F-103) and the lower thread to a rack and pinion. The vessel was suspended in a moist, funnel-shaped chamber, maintained at $37 \pm 0{\cdot}5$°C by a water jacket. The vein was superfused with Krebs solution ($95\%O_2 + 5\%CO_2$) pre-warmed to 37° C ; a roller pump (Extracorporeal) was used to maintain a constant flow of 3 ml/min, and a system of three-way taps enabled the selection of fluids from different reservoirs to be made without disturbing the flow. Variations in flow rate were slight ; for ten consecutive 20 s collections the mean volume was $0{\cdot}98 \pm 0{\cdot}04$ ml.

The rabbit coeliac artery was dissected out from its origin at the aorta for a distance of 2–2·5 cm ; extraneous tissue was carefully removed and a spiral strip 2–3 cm long and 0·3 cm wide was prepared ; the artery was arranged for superfusion in the same way as the vein.

The stimulating electrodes consisted of two platinum wires (0·5 mm diam. and 3 cm long). These were sealed in a glass holder and angled so that the vessel could be positioned lengthwise between, and parallel to, the two wires. The electrodes were adjusted so that they just brushed the walls of the vessel but did not impede mechanical responses ; the superfusate cascaded over both vessel and electrodes. The vessels were stimulated with rectilinear pulses (0·3–0·5 ms duration) for varying periods of time and at frequencies from 0·5 to 10 Hz. The stimuli were of supramaximal strength (12–15 V between the electrodes) at all times. The output from the transducer was displayed on a model 7, Grass polygraph, or on a potentiometric millivolt recorder (Texas Instruments—ServoRiter). The rack and pinion was used to set the initial tension of the vessels at 4 g ; this tension was reset to between 3–4 g after an initial 30 min equilibration period and the experiment started after a further 15–20 minutes.

Uptake studies

Male Sprague-Dawley rats obtained from Charles River (200–300 g) were killed by cervical dislocation and the hearts prepared for coronary perfusion as described by Simmonds & Gillis (1968). The heart was perfused with Krebs solution, prewarmed to 37° C, at a constant flow of 9 ml/minute. Contractility was measured by attaching a clip to the tip of the ventricle and by connecting this to an isometric transducer via a thread and pulley system. A constant equilibration period of 20 min was allowed before starting each experiment ; hearts that became hypodynamic during this period were rejected. 7-(±)-^{3}H-NA (3×10^{-8}M, specific activity 7–10 Ci/mM obtained from New England Nuclear, Corp.) in Krebs solution, was infused for 2, 5 and 10 min periods. Angiotensin (50–500 ng/ml) was present throughout the 20 min equilibration period and during the infusion of noradrenaline in the experimental hearts. After stopping the perfusion the hearts were rinsed, blotted dry, frozen on solid CO_2 and weighed. The hearts were homogenized in 4 + 1 ml of 0·4M perchloric acid containing 0·1% disodium edetic acid. The homogenates were centrifuged at 10,000 *g* for 10 min and the supernatants were retained for alumina chromatography (see below).

The portal vein and coeliac artery were dissected out as described previously. The vein was cut longitudinally into two equal strips ; one strip acted as a control while the other was subjected to drug treatment. The artery was usually divided

longitudinally into three equal strips. The tissue weights varied between 25–40 mg. The strips were first equilibrated in Krebs solution at 37° C for 10 min, and then in Krebs solution with or without angiotensin, for a further 15 minutes. They were then incubated with 20 ng/ml 7-^{3}H-NA (12×10^{-8}M, specific activity 7Ci/mM) in Krebs solution (with or without angiotensin) for 5 or 15 minutes. At the end of the incubation the strips were rinsed, blotted, frozen on solid Co_2 and weighed. The vessels were homogenized in 3 + 1 ml of 15% trichloracetic acid and the superantant prepared as for the rat heart. Uptake of (±)-^{3}H-NA is expressed as the tissue to medium ratio (T/M); that is, $\frac{\text{d.p.m. } ^3\text{H-NA/g tissue}}{\text{d.p.m. } ^3\text{H-NA/ml medium}}$

When transmitter release was to be studied the whole vessel was first equilibrated in Krebs solution at 37° C for 15 min, and then for 1·5 h in Krebs solution containing 20 ng/ml 7-^{3}H-NA (30×10^{-8}M, 5 μCi/ml), and the vessel was transferred to a fresh labelled solution for a further 1·5 hours. The vessel was rinsed in warm Krebs solution and set up for superfusion. At the start of superfusion there was a rapid, initial loss of tritium from the vein, which fell to a basal level of between 1,500–2,000 c.p.m./30 s within 15 min and thereafter declined slowly and linearly, to 400–800 c.p.m./30 s after 5–6 hours. In the following experiments at least 20 min equilibration were allowed before each study. The superfusate was either collected for timed intervals into scintillation vials and the radioactivity directly estimated, or into cooled flasks containing 0·2 ml 2N HCl + 10 mg disodium edetate + carrier NA, normetanephrine, 3,4 dihydroxymandelic acid and 3 methoxy, 4 hydroxymandelic acid (50 μg each). The latter samples were immediately frozen and kept for chromatographic analysis.

Column chromatography

Short alumina columns (British Drug Houses, 1·5 × 0·4 cm) were used to concentrate the NA activity and to separate catecholamines from O-methylated products and tritiated water. Tris buffer (0·2 ml 1 M pH 8·2) and disodium edetate (final concentration, 10 mg/1 ml) were added to the samples and the pH adjusted to 8·4 with NaOH. The samples were immediately applied to the column and recycled once; the column was then washed with 25–30 ml of distilled water and blown dry. The alumina was resuspended in 2 ml of 0·15 N perchloric acid and the catechols eluted; a 0·5 ml aliquot of the eluate was taken for scintillation spectrometry and a further 0·5 ml for determination of endogenous NA by the trihydroxyindole technique (Euler & Lishajko, 1961).

Short Amberlite CG 120 columns (1·5 × 0·8 cm) in the Na^+ form were used to separate noradrenaline and its methylated products from its deaminated products. Disodium edetate (10%, 0·1 ml/2 ml) was added to the sample and the pH was adjusted to 4 with NaOH. The sample was passed once through the column and collected; the column was washed with 20 ml of distilled water and the first 10 ml of this was added to the first effluent (deaminated products). The NA + normetanephrine (NMN) were eluted from the column with 6 ml of 2 N HCl. The recovery of NA and NMN by this procedure was between 75–85 per cent. Values are not corrected for recovery.

Long Amberlite CG 120 columns (14 × 0·4 cm) were used to separate and identify deaminated products, noradrenaline and normetanephrine (Roth & Stone, 1968).

Scintillation spectrometry

All samples were counted in a Packard Scintillation Counter (model 3375) to a constant standard deviation of 1–1·5%. The counting efficiency was 10–20%. The radioactivity was converted to d.p.m. by the technique of automatic external standardization. Eluates from the short Amberlite columns were counted in dioxane containing 5 g of 2,5-diphenyloxazole and 100 g of naphthalene/litre. All other samples were counted in 20 ml of dioxane-ethanol-toluene solvent (1 l. each of toluene, dioxane and ethanol containing 240 g naphthalene, 15 g 2,5-diphenyloxazole and 0·3 g of 1,4-*bis*-2-(4-methyl-5-phenyloxazolyl)-benzene). The Krebs solution had the following composition: NaCl, 118 ; KCl, 4·75 ; $CaCl_2$, 2·54 ; KH_2PO_4, 0·93 ; $MgSO_4$, 1·19 ; $NaHCO_3$, 25·0 ; glucose, 11·1 mM ; disodium edetate, 10 mg/l. ; sodium ascorbate, 20 mg/litre.

Drugs

7-(±)-^{3}H-noradrenaline (New England Nuclear), acetylcholine, cocaine, nicotine hydrogen tartrate, 5-hydroxytryptamine (B.D.H.), angiotensin-amide (CIBA), noradrenaline HCl (Sigma), synthetic lysine vasopressin (CalBiochem), veratrine (alkaloid mixture, CalBiochem), tetrodotoxin (crystalline ; Sankyo-Tokyo and CalBiochem). All drug concentrations are quoted in terms of base (μg or ng/ml).

Results

Effects of angiotensin on the mechanical response of the vein and artery and modification of its effects by drugs

Portal vein. Angiotensin only elicited small contractions of the vein at concentrations above 1–2 μg/ml. These contractions were short-lived, declining to baseline within 60–90 s with angiotensin still present. Repeated doses of angiotensin caused a rapid desensitization to the polypeptide ; this tachyphylaxis was not prevented by incubating the vein with 0·1–10 μg/ml of NA between doses of angiotensin.

Veratrine (1–2 μg/ml), which can be used selectively to potentiate nerve mediated responses in smooth muscle (Paton & Vane, 1963 ; Hughes & Vane, 1967), markedly increased contractions elicited by angiotensin, nicotine and transmural stimulation, but did not affect contractions elicited by noradrenaline, acetylcholine or 5-hydroxytryptamine (Fig. 1). Tetrodotoxin (0·5 μg/ml) abolished the increased response to angiotensin, and the contractions to nicotine and transmural stimulation, while the responses to acetylcholine and 5-hydroxytryptamine were unchanged (Fig. 1). These results were repeated in three different veins.

Angiotensin (0·01–1 μg/ml) increased the height of contractions elicited by transmural stimulation, the potentiation was dose dependent and ranged from 20–100%. Contractions elicited by NA were not potentiated by angiotensin (four experiments). The potentiation was readily observed at stimulation frequencies of 0·5–4 Hz ; the effect was markedly diminished above these frequencies and often was not seen at all. The potentiation by angiotensin was not affected by cocaine (1–4 μg/ml), which itself caused a marked increase in response to transmural stimulation and to NA (Fig. 2). The halftime of decline of the contraction after cessation of transmural stimulation was not significantly altered by angiotensin. In contrast cocaine consistently increased the halftime of decline by 20–50%.

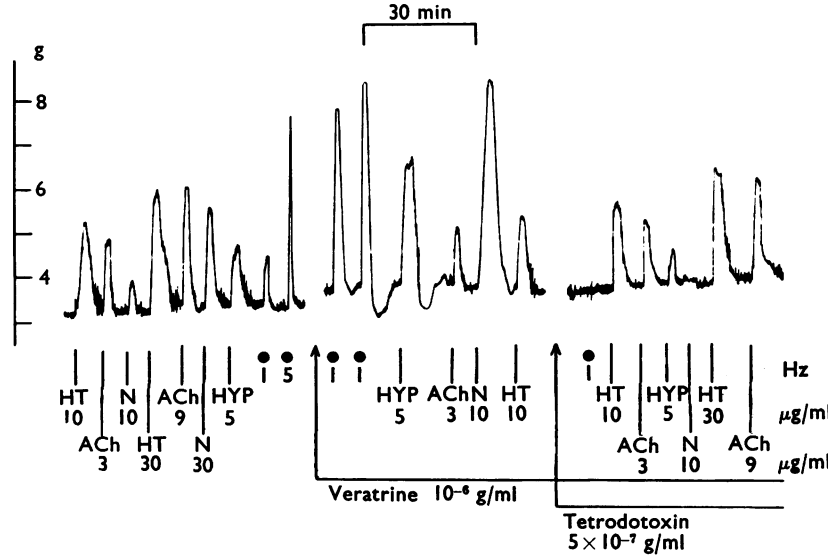

FIG. 1. Effects of veratrine and tetrodotoxin on contractions of rabbit portal vein to directly and indirectly acting drugs. The first panel shows control responses to 5-hydroxytryptamine (5-HT, 10 and 30 μg/ml), acetylcholine (ACh, 3 and 9 μg/ml), nicotine (N, 10 and 30 μg/ml), angiotensin (HYP, 5 μg/ml) and to electrical stimulation (100 pulses at 1 and 5 Hz, 15 V and 0·5 ms). Veratrine (1 μg/ml at first arrow) caused a marked potentiation of the contractions elicited by nicotine, angiotensin and electrical stimulation at 1 Hz, but there was no increase in the responses to acetylcholine and 5-hydroxytryptamine. Tetrodotoxin (0·5 μg/ml at second arrow) added in the presence of veratrine, abolished responses to nicotine and electrical stimulation, and reduced the response to angiotensin to below the initial control response. There was little change in the responses to acetylcholine and 5-hydroxytryptamine. Tissue bath volume=10 ml ; drugs were left in contact with tissue for 60 s before washing out by overflow. Vertical scale=g, time mark=30 minutes.

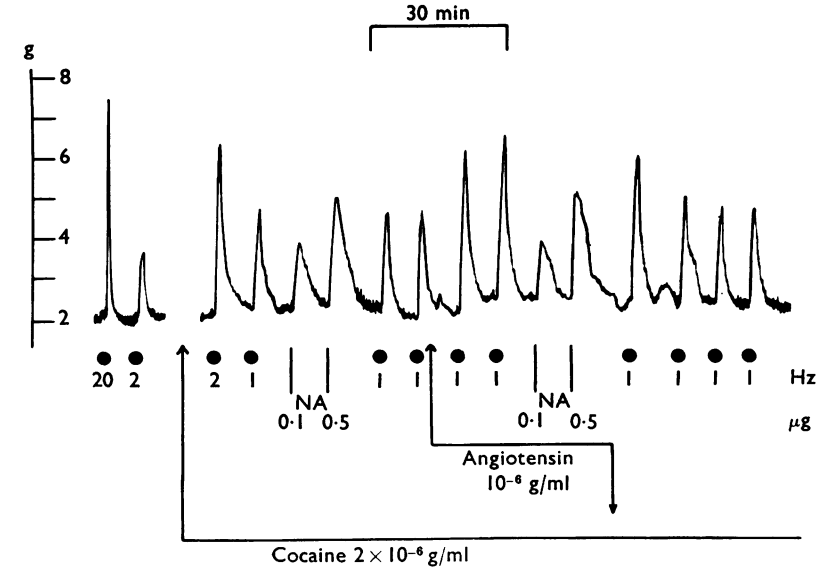

FIG. 2. Potentiation of contractions to electrical stimulation by angiotensin in the presence of cocaine. Rabbit portal vein. First panel, control contractions elicited by electrical stimulation (100 pulses, 2 and 20 Hz, 15 V and 0·5 ms). Cocaine (2 μg/ml at first arrow) increased the height and duration of responses to electrical stimulation, and to noradrenaline (NA, 0·1 and 0·5 μg/10 ml ; control responses before cocaine not shown). Under these conditions constant responses to 1 Hz (sixty pulses) were obtained and then angiotensin (1 μg/ml at second arrow) was added to the bath ; the contraction at 1 Hz was doubled in size but the response to NA was unaltered. When the angiotensin was washed out ($\downarrow$) the contractions to electrical stimulation declined. Vertical scale=g, time mark=30 minutes.

The angiotensin-induced potentiation of responses to transmural stimulation was rapidly reversed when the drug was removed from the medium; however, reintroduction of angiotensin again produced a potentiation. The contractions elicited by transmural stimulation declined somewhat when angiotensin was left in contact with the vein for more than 20–30 min; however, a further decline in contraction height was usually seen when angiotensin was finally removed from the medium.

Coeliac artery. Angiotensin readily elicited contractions in this tissue (5–500 ng/ml); 50% maximal responses were obtained at 50–100 ng/ml; however this value is subject to error since the artery rapidly became tachyphylactic to angiotensin. Veratrine and tetrodotoxin (two experiments) had similar effects on the concentration to angiotensin as in the portal vein.

Low concentrations of angiotensin (0·1–1·0 ng/ml) did not cause appreciable contractile effects, but increased the response to transmural stimulation by 30–100% (5 experiments, 0·5–2 Hz for 30 s). Contractions elicited by NA were not potentiated in these experiments (Table 1). When the contractile effect of higher doses of angiotensin had declined it was possible to demonstrate a 100–150% increase in the size of contractions elicited by transmural stimulation. These increased responses were maintained for up to 30 min at the lower concentrations of angiotensin but frequently declined within 15 min at the higher concentrations. It was frequently noticed that treatment of both vein and artery with angiotensin was followed, after washout of the angiotensin, by a decline in response to transmural stimulation to below the pre-angiotensin control height.

Effect of angiotensin on the uptake of ^{3}H-(±)-NA

Pre-treatment of the portal vein (500 ng/ml), coeliac artery (50 ng/ml) and rat heart (50 and 500 ng/ml) with angiotensin did not reduce the uptake of ^{3}H-(±)-NA at any of the time periods studied, even though angiotensin was present 15 min before, and during exposure to the label (Table 2). In fact there appeared to be an increased uptake of label in the presence of angiotensin when the vein was incubated for 15 min with labelled NA. When the vein was pre-incubated in Krebs alone, and then for 5 min with both labelled NA and angiotensin there was a small

TABLE 1. *Effect of angiotensin on contractions elicited by noradrenaline and transmural stimulation in the coeliac artery*

Treatment	Contraction height (mm)				
	Noradrenaline			Transmural stimulation	
	50	100	200 ng	1 Hz	2 Hz
Control	6	11·5	18·5	7	18
Angiotensin (5 ng/ml)	5	11	19	15	26
Control	5	9	14	5	15
Angiotensin (5 ng/ml)	5	11	17	14	28
Control	6	10·5	17·5	3	12

Typical effect of angiotensin in one rabbit coeliac artery. Artery superfused with Krebs solution at 4 ml/min, 2 g basal tension. Noradrenaline injected directly into superfusate in volumes of less than 50 μl. Electrical stimulation for 60 s at 1 and 2 Hz, 15 V across tissue at 0·5 ms. The stimuli were randomized and each tabulated value represents the mean of two observations. Angiotensin (5 ng/ml) added to the Krebs, increased the response at 1 Hz by 114% and at 2 Hz by 45%, but did not affect noradrenaline-induced contractions.

but significant reduction in uptake of label. The uptake of label was not significantly reduced when the same procedure was followed with the coeliac artery (Table 2).

For comparison, the uptake of ^{3}H-(±)-NA (20 ng/ml for 5 min) was determined in two veins pre-incubated for 15 min in the presence of cocaine (500 ng/ml) and compared with untreated veins. The tissue/medium (^{3}H) ratios were found to be 0·4 and 0·6 in the cocaine treated veins, and 2·20 and 2·65 in the controls. Thus cocaine has a marked inhibitory effect on uptake of ^{3}H-NA in the portal vein.

Release of ^{3}H-NA from the rabbit portal vein

Identity of ^{3}H-labelled compounds in vein and superfusate. The metabolite distribution and specific activity of the ^{3}H-NA present after incubation for 3 h of the vein with label were determined after rinsing the vein in amine-free Krebs solution at 37° C for 20 minutes. Alumina separation followed by long Amberlite column chromatography was used to separate the metabolites. Endogenous NA was determined from an aliquot of the alumina column extract. Over 93% of the tissue (^{3}H) activity was associated with intact noradrenaline (Table 3). The specific activity of the tissue noradrenaline was between 30 and 45% of that in the incubating medium (Table 3).

TABLE 2. *Effect of angiotensin on the uptake of ^{3}H(±)-NA*

Tissue	(*n*)	Incubation or perfusion time	Tissue/medium ^{3}H ratio ±S.E.M. Control	Angiotensin
Rabbit portal vein	(3)	5 min	2·72±0·3	2·85±0·22 (a)
	(3)	15	6·10±0·2	7·6±0·3* (a)
	(4)	5	2·50±0·2	1·9±0·3† (b)
Rabbit coeliac artery	(4)	5	2·80±0·4	2·9±0·2 (c)
	(4)	5	2·80±0·4	2·5±0·3 (d)
Rat perfused heart	(4)	2	1·68±0·1	2·0±0·2 (a)
	(4)	5	3·69±0·55	3·62±0·3 (a)
	(4)	10	7·74±0·3	8·2±0·4 (a)

* Significantly greater than control ($P>0·05$); † significantly smaller than control ($P<0·05$).
The rabbit portal vein, coeliac artery and rat hearts were prepared as decribed in **Methods.** The vascular tissues were pre-incubated for 15 min in Krebs alone, or in Krebs+angiotensin. They were then incubated with 20 ng/ml ^{3}H-(±)-NA (specific activity 7 Ci/mM) in Krebs, with or without angiotensin for 5 or 15 minutes. The rat perfused hearts were treated similarly but the noradrenaline concentration was reduced to 5 ng/ml in these experiments (specific activity 7 Ci/nmol). Treatment code: a, angiotensin, 500 ng/nl, present in both the pre-incubated period and during incubation with ^{3}H-NA; b, angiotensin, 500 ng/nl, only present during incubation with ^{3}H-NA; c, identical to a but angiotensin concentration of 50 ng/ml; d, as b, angiotensin 50 ng/ml.

TABLE 3. *Specific activity and metabolite distribution in portal vein after incubation for 3 h with ^{3}H-(±)-NA*

Vein	Specific activity d.p.m./ng NA	% NA	Chromatography % NMN	% Deaminated
1	27,606	94·3	1·4	4·3
2	27,747	95·5	1·2	3·3
3	17,769		(not analysed)	

The veins were incubated for 3 h with 5 μCi/ml of labelled noradrenaline (specific activity 60,000 d.p.m./ng). The endogenous noradrenaline content of the veins was determined after alumina column separation. The alumina eluates of two veins were passed through long Amberlite (CG-120) columns in order to determine the complete metabolite distribution.

The composition of the ^3H efflux under basal and stimulated conditions was determined after collecting the superfusate for 3 min immediately before and then during transmural stimulation. These samples were treated as described in **Methods** and analysed for NA, NMN and deaminated products. The basal (^3H) release consisted mainly of deaminated products and of a small proportion of ^3H-NA and ^3H-NMN (Table 4). When the vein was stimulated at 2 Hz for 3 min there was a marked increase in the efflux of ^3H-NA and a smaller increase in the efflux of labelled, deaminated and O-methylated products (Table 4). When the frequency of stimulation was increased to 5 and 10 Hz, a similar pattern was seen although the total efflux was much greater.

Effect of angiotensin on the release of ^3H-NA. In the following experiments ^3H-NA and ^3H-labelled methylated products were separated from deaminated products by the short Amberlite column procedure. Samples were collected for 1·5 min immediately before, 1 min during, and 0·5 min after stimulation. Figure 3 shows the efflux patterns obtained at rest and during stimulation at 2 Hz for 60 seconds. There was a marked increase in labelled NA + NMN release during each stimulation period. This increase varied from 40 to 50% at the start of the experiment, to 80–100% after two or three stimulation periods when the basal release of NA + NMN had declined somewhat. The total release of NA + NMN declined with each stimulus period; this was most marked during the first two or three stimulation periods, thereafter the decline was much slower.

Angiotensin (200 ng/ml, three experiments) increased the efflux of labelled NA + NMN during stimulation but did not alter the basal efflux (Fig. 3). This increase was associated with an increased tension response. When the vein was reperfused with angiotensin-free Krebs solution there was an immediate fall in both the amine efflux and contraction during electrical stimulation.

Similar results were obtained when cocaine (two experiments, 4 μg/ml) was added to the Krebs solution (Fig. 4). This concentration of cocaine was previously

TABLE 4. *Metabolite composition of ^3H efflux from portal vein under stimulated and non-stimulated conditions*

		Chromatographic analysis of perfusate					
		Noradrenaline		Normetanephrine		Deaminated	
	Conditions	d.p.m.	% Total	d.p.m.	% Total	d.p.m.	% Total
	Basal	3,530	5	4,460	6	64,000	89
	2 Hz	12,260	14	4,740	5	73,000	81
(A)	Basal	3,300	5	4,010	5	66,120	90
	5 Hz	36,200	29	5,940	5	82,500	66
	Basal	4,530	8	1,990	4	45,230	88
	10 Hz	54,000	33	6,370	4	103,950	63
	Basal (control)	3,200	6	970	2	43,240	92
	2 Hz (control)	31,700	35	5,830	6	54,720	59
(B)	Basal (angiotensin)	1,550	4	1,850	5	33,720	91
	2 Hz (angiotensin)	40,080	47	5,390	6	39,250	47

The effluent from the vein was collected into cooled flasks containing 0·2 ml 2N HCL + 10 mg EDTA + carrier noradrenaline, normetanephrine and 3·4 dihydroxymandelic acid (50 μg each). Three minute collections were made immediately before stimulation (basal) and during electrical stimulation for 3 min (2, 5 and 10 Hz). The samples were then taken through the long Amberlite column procedure to separate noradrenaline, normetanephrine and deaminated products. (A), metabolite pattern obtained in one experiment from an untreated vein; (B), vein treated with cocaine (4 μg/ml), metabolite pattern before and during treatment with angiotensin (500 ng/ml).

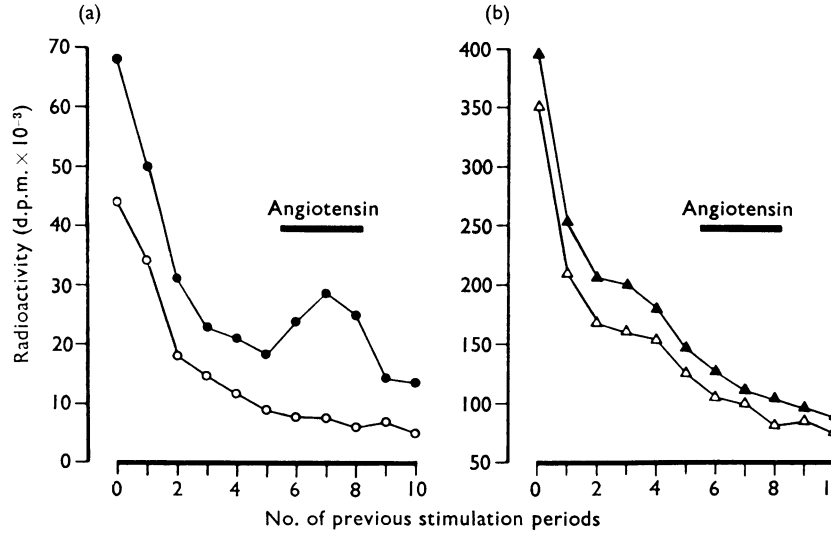

FIG. 3. Effect of angiotensin on the basal and stimulated efflux of labelled noradrenaline and its metabolites from the portal vein. Typical experiment with vein loaded with (±)-^{3}H-NA as described in **Methods.** The vein was stimulated for 1 min at 2 Hz every 7 minutes. Two samples were collected each time, these consisted of (1) 2·5 min basal sample immediately preceding stimulation, and (2) 2·5 min sample which included the whole of the stimulation period and the 1·5 min following it. The metabolites were separated on short Amberlite columns as described in **Methods.** Angiotensin (200 ng/ml), at horizontal bars, increased the stimulated efflux of NA+NMN (●) but not the basal effluxes of NA+NMN (○) and of the deaminated products (△), or of the stimulated efflux of deaminated products (▲). Ordinates: (^{3}H) activity (d.p.m.); (a) NA+NMN, (b) deaminated products.

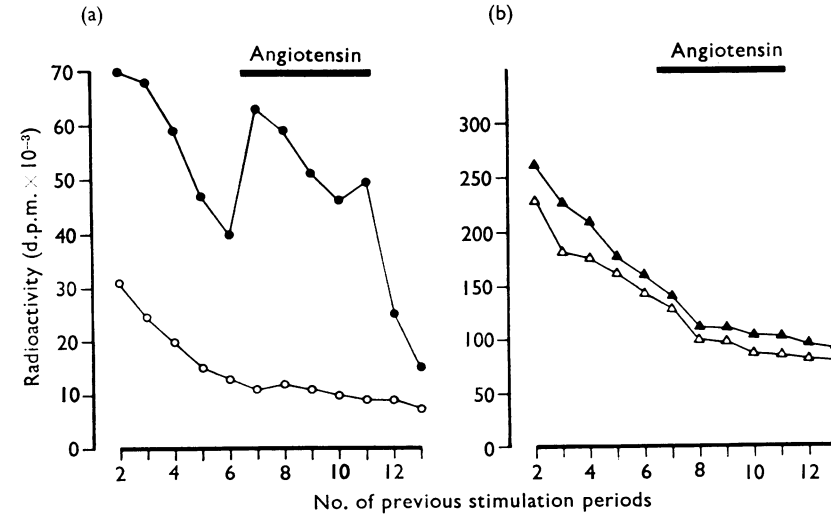

FIG. 4. Effect of angiotensin on the basal and stimulated efflux of labelled noradrenaline and its metabolites from portal vein treated with cocaine. Typical experiment with same conditions as in Fig. 3 but with cocaine (4 μg/ml) added to Krebs superfusate. The stimulated output of NA+NMN was consistently greater in the presence of cocaine, (note that Fig. 4 starts after two previous stimulation periods) but angiotensin (200 ng/ml, at horizontal bars) still enhanced the stimulated output of NA+NMN (●). The basal outputs of NA+NMN (○) and deaminated products (△), and the stimulated outputs of deaminated products (▲) were unaffected by angiotensin. Ordinates: (^{3}H) activity (d.p.m.); (a) NA+NMN, (b) deaminated products.

found to give a maximal potentiation of the contraction elicited by transmural stimulation. Under these conditions the efflux of labelled NA+NMN, during stimulation, was double that seen in the absence of cocaine. However, angiotensin (200 ng/ml) was still able to further increase the output of labelled amine by 60–100% (Fig. 4).

Effects of angiotensin on total ^3H release. Once the pattern of metabolite release had been established it was possible to carry out further confirmatory experiments by directly measuring ^3H efflux without further separation. Electrical stimulation for 1 min at 0·5, 1, 2, 5 and 10 Hz was associated with an increased release of ^3H into the superfusate. When the vein was stimulated at the same frequency for 1 min at 7 min intervals, the total ^3H released declined with each successive stimulus. However, when the amount released during stimulation was expressed as a percentage increase above basal release (determined in the minute preceding stimulation) the values did not show such a great disparity. The percentage increase above basal varied from 10 to 30% at 0·5–1 Hz, and from 150–300% at 10 Hz.

Angiotensin (10–400 ng/ml) had no effect on the basal efflux of ^3H but markedly increased the stimulated efflux at 0·5, 1 and 2 Hz (Table 5, eight experiments) but not at or above 5 Hz (three experiments). Higher concentrations of angiotensin (0·5–2 μg/ml) caused a small increase in the basal ^3H efflux (6–20%) in three out of five experiments, but this effect was only seen during the first 1–2 min of the angiotensin infusion. The potentiating effect of angiotensin appeared to reach a maximum at 0·5–1·0 μg/ml, the increase in ^3H efflux during stimulation was between 100 and 150% at these concentrations.

When low concentrations of angiotensin (10–500 ng/ml) were infused after the start of a period of transmural stimulation (2 Hz), the vein contracted further even though the contraction due to electrical stimulation had reached a plateau. This contractile response to angiotensin was associated with an increase in the ^3H efflux (Fig. 5). These concentrations of angiotensin never elicited a contraction in the absence of electrical stimulation.

Four further experiments were carried out in the presence of cocaine (4 μg/ml). The release of ^3H during stimulation was consistently increased in the presence of

TABLE 5. *Effect of angiotensin on total (^3H) efflux during transmural stimulation*

Treatment	(^3H) Activity (d.p.m.) Basal	Stimulated	Increase due to stimulation		Contraction Height (mm)
None	20,800	35,200	14,400	(69%)	11
	20,600	34,700	14,100	(68%)	11
Angiotensin 200 ng/ml	19,300	39,500	20,500	(106%)	14
	18,900	40,000	21,100	(109%)	15
None	19,000	31,900	12,900	(68%)	11
	18,800	29,330	10,530	(56%)	9
Angiotensin 200 ng/ml	17,800	34,550	16,750	(94%)	12
	17,100	33,860	16,760	(98%)	12
	16,500	26,400	9,900	(60%)	9
None	16,200	25,110	8,910	(55%)	8
	16,000	24,500	8,500	(53%)	8

Single typical experiment showing that angiotensin causes a reproducible increase in total (^3H) output during transmural stimulation (2 Hz for 1 min every 7 min). Vein loaded with ^3H-NA as described in **Methods.** Each value represents counts detected in 2 min samples (1) immediately before stimulation and (2) during and for 1 min after stimulation. The figures in brackets represent the percentage increase in radioactivity above basal values during stimulation.

cocaine thus confirming the earlier experiments involving separation of the metabolites. The stimulated efflux was usually increased by 100% with cocaine; angiotensin (500 ng/ml) also caused a similar increase in efflux during stimulation under these conditions.

Time course of ^3H release during electrical stimulation. In three experiments the contraction was recorded at fast paper speed and consecutive 20 s samples of the superfusate were collected before, during and after stimulation for 1 min at 2 Hz. The peak efflux of ^3H release occurred between the last 20 s of stimulation and the 20 s following the end of stimulation (Fig. 6). The efflux was consistently higher in the presence of angiotensin (500 ng/ml) except during the first 20 s of stimulation (Fig. 6).

Effects of transmural stimulation and angiotensin on release of ^3H from the coeliac artery

A detailed examination of the metabolite pattern was not made with this tissue. However, two preliminary experiments indicated that the basal ^3H release consisted mainly of deaminated products, while there was a marked increase in the efflux of labelled NA + NMN during transmural stimulation. In the following experiments the ^3H efflux was directly determined without prior separation.

Angiotensin-induced contractions (10–100 ng/ml, four experiments) were associated with a marked increase in ^3H efflux. The increased efflux and the associated contraction declined when the angiotensin infusion was continued for more than 1–2 min (Fig. 7). The ^3H efflux during electrical stimulation was increased by 200–300% in the presence of these concentrations of angiotensin. Lower concentrations

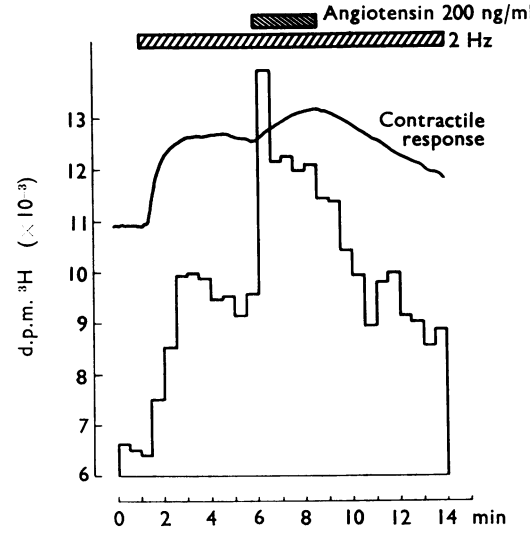

FIG. 5. Effect of angiotensin on total ^3H efflux and contractility of portal vein during continuous electrical stimulation. Vein loaded with (±)-^3H-NA as described in **Methods.** Total tritium efflux determined in consecutive 30 s samples. Electrical stimulation (2 Hz) elicited a contraction and an increased efflux of (^3H) which reached a maximum and then slowly declined. Introduction of angiotensin (200 ng/ml) increased both the efflux of label and caused a further contraction of the vein. Ordinate: total tritium efflux. A tracing of the mechanical response is superimposed on the histogram.

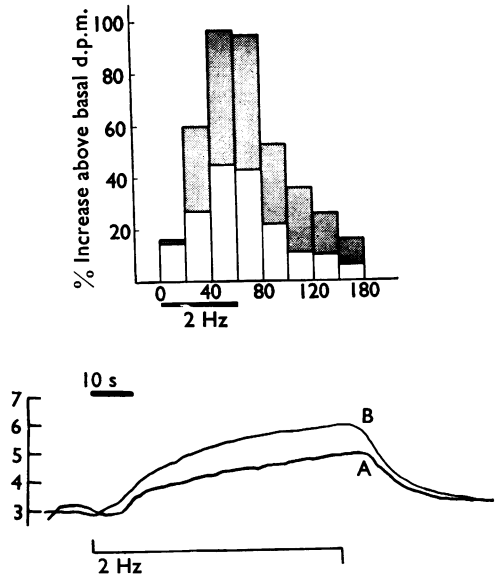

FIG. 6. Effect of angiotensin on the pattern of ^3H efflux and on the contraction at fast paper speed during electrical stimulation. Upper panel: ordinate, percentage increase in radioactivity above basal during stimulation at 2 Hz for 1 min; 20 s samples; (□) control efflux; (▨) increase in efflux seen in presence of angiotensin (200 ng/ml). Lower panel: mechanical response at fast paper speed; A, control response, B, response in the presence of angiotensin (200 ng/ml); ordinate, g; Time mark, 10 seconds. Not same scale as upper panel.

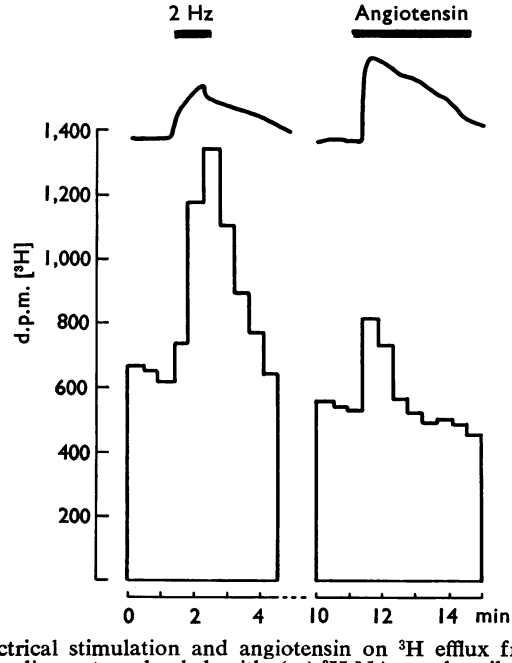

FIG. 7. Effect of electrical stimulation and angiotensin on ^3H efflux from the rabbit coeliac artery. Superfused coeliac artery loaded with (±)-^3H-NA as described in **Methods.** Consecutive 30 s samples collected before and during electrical stimulation (2 Hz for 1 min at first bar) and angiotensin (20 ng/ml at second bar). Note the much larger increase in ^3H efflux during electrical stimulation despite the smaller contractile response compared to angiotensin. Ordinate: total tritium output (d.p.m.). A tracing of the mechanical response is superimposed on the histogram, identical time scales.

of angiotensin (0·1–1 ng/ml; five experiments) caused a small (5–10%), transient increase in the basal ^{3}H efflux and increased the stimulated ^{3}H efflux by 30–150%.

Effects of other stimuli on the release of ^{3}H from the rabbit portal vein and coeliac artery

There was no increase in the basal ^{3}H efflux when the vein and artery were passively stretched by increasing basal tension by 1–3 g. Vasopressin (0·1–1·0 μg/ml) did not affect the efflux of ^{3}H under basal or stimulated conditions in the portal vein, neither did it alter the contractions elicited by electrical stimulation. Vasopressin (0·1–1·0 μg/ml, two experiments) elicited small contractions of the coeliac artery, but only small and irregular increases in ^{3}H efflux were seen.

5-Hydroxytryptamine (1–10 μg/ml, three experiments) caused a long lasting contraction of the portal vein and increased the ^{3}H efflux by 30–40% above basal levels during the contraction period. Noradrenaline (0·1–1·0 μg/ml) increased the total ^{3}H efflux by a factor of 2–20. Acetylcholine (1–10 μg/ml) had similar contractile effects to 5-HT but only increased the ^{3}H efflux by 5–8% above basal levels.

Discussion

The method of transmural stimulation has previously been validated as a means for exciting the sympathetic nerve supply to the rabbit portal vein (Hughes & Vane, 1967). Incubation of the vein with labelled NA leads to the accumulation of label within the tissue, and over 90% of the radioactivity retained in this tissue is associated with intact NA. The label appears to be firmly bound and is presumably associated with sympathetic nerves. Direct evidence for this has been obtained using the combined techniques of autoradiography and electronmicroscopy (Hughes, J. & Bloom, F. E., unpublished results). Transmural electrical stimulation of the vein elicits contractions which are associated with an increase in the efflux of intact ^{3}H-NA. Mechanical stimulation alone does not increase the efflux of label.

O-Methylation and oxidative deamination clearly play a role in the metabolism of ^{3}H-NA in the vein, and the efflux of these metabolites is increased during electrical stimulation, particularly at the higher frequencies of stimulation. Although the observation was not pursued, it was also noticed that in the presence of cocaine there appeared to be a greater efflux of O-methylated label during stimulation. In contrast to stimulus-induced release, the basal release of label was almost entirely composed of deaminated products. At present we are unable to say whether this reflects intra- or extra-neuronal metabolism of NA. Langer (1968) has obtained a somewhat different pattern of metabolite release with the isolated nictitating membrane of the cat. This difference may be due to differences in the rate of diffusion of NA from the tissues and hence differences in the times of exposure to metabolizing enzymes.

Considerable care must be taken in interpreting the effects of agents which potentiate responses to sympathetic stimulation. This particularly applies when effects of NA uptake may be involved, since confusion may arise if the potentiating agent is also capable of releasing NA. This appears to be the case for angiotensin. The pharmacological results with veratrine and tetrodotoxin suggest that angiotensin can directly stimulate adrenergic nerves, albeit weakly; this was confirmed

directly when it was found that angiotensin increased the efflux of label from the portal vein and coeliac artery. This conclusion is in agreement with the results of other workers (Distler, Liebau & Wolf, 1965 ; Peach & Ford, 1968 ; Kiran & Khairallah, 1969).

The initial uptake of ^{3}H-NA was not reduced by angiotensin, except when angiotensin was simultaneously administered with the label in the portal vein. This effect could not be demonstrated in the rat heart, rabbit portal vein or coeliac artery when they were incubated with angiotensin before the label, that is under conditions in which we were able to demonstrate increased responses to sympathetic stimulation. It is likely that the apparent inhibition of uptake here, and in other work (Palaić & Khairallah, 1967 ; Panisset & Bourdois, 1968 ; Peach *et al.*, 1969) is due to the initial release of label by angiotensin. It is perhaps significant that Panisset & Bourdois administered angiotensin and ^{3}H-NA simultaneously and could only demonstrate an apparent inhibition of uptake during the first 3 min of the experiment. The release of endogenous NA by angiotensin, although not demonstrated by us, might also explain the apparent increase in uptake seen in our experiments when the vein was initially treated with angiotensin before incubation with the ^{3}H-NA.

Angiotensin did not enhance responses to exogenous NA. Further, cocaine, which markedly inhibited ^{3}H-NA uptake and increased the responses to electrical stimulation and to exogenous NA, did not prevent the potentiating action of angiotensin. This supports the view that this potentiating effect of angiotensin is unrelated to any inhibition of NA uptake. Pals & Masucci (1968) were also unable to demonstrate any effect of angiotensin on the uptake of NA. Indeed it would be strange if angiotensin did have such an effect since it is known to potentiate responses to tyramine (McCubbin & Page, 1963 ; Day & Owen, 1969), an effect not normally associated with inhibitors of NA uptake.

In both the portal vein and coeliac artery the increased response to electrical stimulation was associated with an increase in the efflux of ^{3}H-NA and ^{3}H-NMN, but not of deaminated metabolites. The potentiation was reversed on washing out the angiotensin and could be demonstrated many times in the same preparation as well as in preparations treated with cocaine. The coeliac artery was more sensitive to the effects of angiotensin than the vein, but the vein proved more robust and consistent in its responses and was chosen as the more suitable test tissue. The increased ^{3}H efflux was apparent throughout the stimulation period apart from the first 15–25 s of stimulation. This was strange since the tension response showed a quicker onset of action as well as an increased maximum in the presence of angiotensin. It is possible that the observed ^{3}H efflux does not reflect the whole pattern of endogenous transmitter release due to an incomplete mixing of label with neuronal NA, this point is now being investigated.

The angiotensin-induced potentiation does not appear to be due to an inhibition of metabolizing enzymes since (a) the total output of ^{3}H was always increased, and (b) there was no observable change in the metabolite pattern in the presence of angiotensin. We must therefore conclude that angiotensin somehow increases the output of transmitter in response to electrical stimulation. This is unlikely to be due to a weak depolarizing action of angiotensin summating with the electrical stimulus since supramaximal stimuli were always employed. It may be that angiotensin is able to modify the ionic balance in the sympathetic nerve terminals in

such a way as to facilitate the release of NA. Angiotensin has been shown to increase sodium and water transfer in the rat isolated jejunum (Crocker & Munday, 1967, 1970), and it may be that this peptide plays a role in modulating several membrane transport processes.

We must emphasize that our results do not provide any evidence for the hypothesis that the direct release of noradrenaline from sympathetic nerves by angiotensin contributes significantly to the contraction of vascular smooth muscle by the peptide. There is obviously a tendency for angiotensin to release small amounts of the sympathetic transmitter, but the effect is small and requires high concentrations of the peptide. We suggest that the facilitation of noradrenaline release is a major effect of the interaction between angiotensin and the sympathetic nerve ending, and that this effect depends on the presence of sympathetic nerve activity.

Both NA and 5-hydroxytryptamine caused an appreciable release of ^{3}H from the labelled tissues ; however, unlike angiotensin there is no pharmacological evidence for an indirect action of these drugs in this or previous work (Hughes & Vane, 1967). It is likely that both noradrenaline and 5-hydroxytryptamine can release ^{3}H-NA by exchange with labelled tissue stores. Acetylcholine has little or no sympathomimetic activity in the portal vein and neither does it appear to exchange with labelled NA.

Angiotensin appears to belong to a unique class of naturally occurring hormones able to modify adrenergic function via a neurogenic mechanism, and the only one so far shown that increases transmitter release. Further work is required to see if this effect has physiological or pathological implications. Angiotensin can also accelerate noradrenaline synthesis in various tissues (Boadle, Hughes & Roth, 1969). At present, we are unable to say whether this effect and the facilitation of transmitter release are causally related, but it is obvious that the two effects would be complementary in enhancing sympathetic nerve function.

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