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Acceleration of catecholamine biosynthesis in sympathetically innervated tissues by angiotensin-II-amide

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Summary

1. The effect of angiotensin-II-amide on the biosynthesis of catecholamines (CA) has been studied in a number of isolated tissues *in vitro*.

2. Angiotensin increased the synthesis of CA from ^{14}C-tyrosine in guinea-pig atria and portal vein, in rat vasa deferentia and the rabbit portal vein.

3. Angiotensin had no effect on synthesis of CA from ^{14}C-labelled DL-DOPA.

4. The conditions required to demonstrate an increased synthesis were critical with respect to incubation time and angiotensin concentration. Effects were most readily apparent after incubation for 1 h with concentrations of angiotensin ranging from 10^{-9} to 10^{-7}M. Higher concentrations caused a significant reduction in synthesis.

5. An increased release of newly synthesized CA into the incubation medium was sometimes seen in the presence of angiotensin. However, there was no correlation between increased synthesis and release of CA.

6. Angiotensin was rapidly destroyed when incubated with guinea-pig or rat tissues in Krebs solution. The increase in CA synthesis was only apparent at a time when the incubation medium could have contained only a fraction of the original angiotensin activity.

7. It is concluded that the effect of angiotensin is not due to increased release of noradrenaline (NA) or to inhibition of NA uptake into nerves. It is possible that angiotensin may influence the activity of tyrosine hydroxylase or its cofactors by an as yet unknown mechanism.

Introduction

Among the numerous effects which have been ascribed to the polypeptide hormone angiotensin are many that involve an action on the sympathetic nervous system. For example, angiotensin stimulates certain centres in the central nervous system causing an increase in sympathetic outflow (Bickerton & Buckley, 1961; Halliday & Buckley, 1962; Buckley, Bickerton, Halliday & Kato, 1963; Smookler, Severs, Kinnard & Buckley, 1966; Severs, Daniels & Buckley, 1967; Lowe & Scroop, 1969; Buckley, 1972); it causes a release of catecholamines (CA) into the circulation by stimulating the adrenal medulla (Feldberg & Lewis, 1964; 1965; Piper & Vane, 1967; Staszewska-Barczak & Vane, 1967; Reit, 1972) and it also

has a direct excitatory action on sympathetic ganglion cells (Lewis & Reit, 1965; 1966; Panisset, Biron & Beaulnes, 1966; Trendelenburg, 1966; Farr & Grupp, 1967; Reit, 1972). In addition this polypeptide has been observed to potentiate vasoconstrictor responses produced by electrical stimulation of sympathetic neurones (Zimmerman & Gomez, 1965; Panisset & Bourdois, 1968; Zimmerman & Gisslen, 1968; Hughes & Roth, 1969; 1971) or by indirectly acting sympathomimetic amines (McCubbin & Page, 1963; Schmitt & Schmitt, 1967; Day & Owen, 1969) and to increase the release of noradrenaline (NA) on nerve stimulation (Zimmerman & Whitmore, 1967; Hughes & Roth, 1969; 1971; Starke, Werner & Schümann, 1969; Starke, 1970; Starke, Werner, Hellerforth & Schümann, 1970; Zimmerman, 1972). Claims have also been made that angiotensin inhibits the axonal uptake of NA into sympathetically innervated tissues (Palaic & Khairallah, 1967; Peach, Bumpus & Khairallah, 1969, Khairallah, 1972) but these reports have not been confirmed by investigations in this (Hughes & Roth, 1971) or in other laboratories (Pals, Fulton & Masucci, 1968; Pals & Masucci, 1968; Panisset & Bourdois, 1968; Chevillard & Alexandre, 1970; Schümann, Starke, Werner & Hellerforth, 1970).

This paper describes a further action of angiotensin, namely an increase in the biosynthesis of NA from tyrosine. The conditions needed to demonstrate this effect are described in detail, and the possible mechanism of the angiotensin effect are discussed. Some of these results have already been published in preliminary form (Boadle-Biber, Hughes & Roth, 1969).

Methods

The effect of angiotensin-II-amide on synthesis of CA in a variety of sympathetically innervated tissues was investigated. These tissues included vasa deferentia from Sprague Dawley rats, vasa deferentia, atria, portal vein and coelic artery from male albino guinea-pigs, and portal vein and coelic artery from male albino rabbits. In addition bovine splenic nerves obtained from freshly slaughtered steers were used as a source of pure sympathetic nerve axons.

Tissues were dissected out rapidly and incubated at 37° C in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit bicarbonate solution containing tyrosine-^{14}C, specific activity 10 mCi/mmol in a final concentration of 5×10^{-5}M. The tyrosine-C^{14} (L-tyrosine-C^{14}, uniformly labelled, specific activity 352 mCi/mmol, New England Nuclear Corp.) was purified before use and the specific activity adjusted to 10 mCi/mM in the manner already described (Boadle-Biber, Hughes & Roth, 1970). In one experiment on guinea-pig atria DL-3,4-dihydroxyphenylalanine-2-C^{14} (DOPA) specific activity 2·94 mCi/mmol, final conc. $2{\cdot}54 \times 10^{-4}$M (New England Nuclear Corp.) was used as substrate instead of tyrosine-C^{14} in order to by-pass the rate-limiting step in CA synthesis, tyrosine hydroxylation. Angiotensin-II-amide (Hypertensin, CIBA; Schwarz) was added to the media of experimental tissues in the desired concentration just before the start of the incubation and the CA synthesis in these tissues was then compared with that in untreated controls. The effect of another polypeptide, vasopressin (Calbiochem), was also tested on CA synthesis in guinea-pig atria.

At the end of the incubation period the tissues were blotted rapidly and frozen on solid carbon dioxide. They were then weighed, homogenized in ice cold 15% (w/v) trichloracetic acid and centrifuged for 15 min at 20,000 *g* in a refrigerated

Sorval centrifuge. The supernatant was taken for the isolation of catechols by the alumina column procedure (Boadle-Biber *et al.*, 1970). A sample of the acid eluate from the alumina columns was counted by liquid scintillation spectrometry and the remainder was used for measurement of endogenous NA by the trihydroxyindole method. In one tissue, guinea-pig atria, the distribution of the newly formed catechols in control and angiotensin-treated tissues between NA, dopamine (DA), and deaminated catechol metabolites was determined by Amberlite CG-120 column chromatography of pooled acid eluates from the alumina columns. The tyrosine-C^{14} content of the tissues was determined by liquid scintillation counting of samples of the effluent from the alumina columns. ^{14}C-catechols released into the bath fluid were also analysed by alumina chromatography. The details of these procedures have already been described at length (Boadle-Biber *et al.*, 1970; Boadle-Biber & Roth, 1972). Values of CA synthesized are expressed as (dpm)/g fresh tissue. A correction has been made for recovery of NA from the alumina columns. This was 80%.

Release of labelled catechols

A study was also made of the effect of angiotensin-II-amide on the release of newly synthesized and exogenously supplied NA from guinea-pig atria with time. Tissues were loaded for 1 h with ^{3}H-NA ((−)-noradrenaline-7-[H^3], specific activity 7–9 mCi/mmol, final concentration 20 ng/ml) as already described (Hughes & Roth, 1971) or were incubated with ^{3}H-tyrosine (L-tyrosine-3,5-H^3, specific activity 25 mCi/mM, final concentration to 10 μC/ml; New England Nuclear Corp.) at 37° C for 30 min to allow formation of ^{3}H-NA.

Atria loaded with newly synthesized ^{3}H-NA were incubated in 5 ml Krebs-Henseleit bicarbonate solution for a 5 min period and then transferred to fresh Krebs-Henseleit solution (5 ml) for 4 consecutive 5 min periods. The incubation medium was then changed to Krebs-Henseleit bicarbonate solution containing angiotensin (5×10^{-7}M) and the sequence of incubations was repeated. The 10 bath fluids (5 ml each) were then analysed by the alumina absorption technique for ^{3}H-catechol compounds. Atria which had been loaded with exogenous ^{3}H-NA, were superfused according to the procedure of Hughes & Roth (1971). A control superfusion in Krebs solution was carried out for 30 min and this was then followed by superfusion with Krebs solution containing angiotensin (5×10^{-7}M) for a further 20 minutes. Samples were collected continuously for one minute periods directly into scintillation vials and counted.

Inactivation of angiotensin

Experiments were carried out to determine whether there was an appreciable loss of angiotensin activity when isolated atria or vasa deferentia were incubated in Krebs solution containing angiotensin. The tissues were incubated at 37° C in 5 ml of Krebs solution containing 250 ng angiotensin. The incubation was stopped at various times (5, 10, 30 and 60 min) and the angiotensin activity was estimated by bioassay on the isolated rat colon treated with 5 μg/ml propranolol (Regoli & Vane, 1964).

Results

Effect of angiotensin-II-amide on synthesis of catecholamines from tyrosine

Angiotensin-II-amide was found to increase CA synthesis by as much as 57% in a variety of sympathetically innervated tissues which are listed in Table 1. In these experiments the tissues were incubated with tyrosine-C^{14} for a period of 1 hour. The concentrations of angiotensin shown in Table 1 are those that gave a maximal increase in CA synthesis. There was no increase in the uptake of labelled tyrosine by the angiotensin-treated tissues compared with the controls. A large acceleration of synthesis was observed in the portal veins of both guinea-pig and rabbit treated with angiotensin (5×10^{-7}M). However, in another vascular tissue, the rabbit coeliac artery, no such acceleration was seen even when a number of different concentrations of angiotensin were tested (2×10^{-7}; 10^{-9}; 10^{-10}M) although in one experiment a significant increase in the release of newly synthesized CA was observed at 10^{-9}M. CA synthesis in guinea-pig coeliac artery also failed to respond to angiotensin (5×10^{-7}M). Formation of CA in vasa deferentia from rabbit and guinea-pig tissue was unaltered by angiotensin treatment. In this instance the guinea-pig tissue was tested with a wide range of concentrations of angiotensin (5×10^{-7}; 10^{-7}; 5×10^{-8}; 5×10^{-9}; 10^{-9}M) whilst the rabbit vas deferens was tested with a single concentration of 5×10^{-7}M. When CA synthesis was examined in bovine splenic nerve, a tissue which is devoid of nerve terminals and effector organs, angiotensin was found not to have any stimulatory effect. In fact at a concentration of 5×10^{-7}M a significant inhibition of CA synthesis was observed. Vasopressin in concentrations of 5×10^{-7}M and 5×10^{-8}M had no effect on CA synthesis in guinea-pig atria, a tissue which showed an acceleration of CA formation in the presence of angiotensin (5×10^{-7}M).

Variation of noradrenaline synthesis

The effects of angiotensin-II-amide on CA biosynthesis in these sympathetically innervated tissues and sympathetic neurones show that there was much variability in the responses obtained. The most reproducible results were seen with rat vas

TABLE 1. *Synthesis of ^{14}C-catechols from ^{14}C-tyrosine in some sympathetically innervated tissues incubated in the presence and absence of angiotensin for* 1 *hour*

Tissue	Treatment	n*	^{14}C-catechols ((dpm)/g) $\times 10^{-3}$ ±S.E.M.	% Increase ^{14}C-catechols
Guinea-pig atria	Control	(9)	39·9 ±2·5	
	Angiotensin 5×10^{-7} M	(4)	52·1 ±1·3[a]	+31
Guinea-pig portal vein	Control	(4)	16·4 ±2·2	
	Angiotensin 5×10^{-7} M	(8)	24·8 ±1·6[b]	+51
Rabbit portal vein	Control	(4)	16·2 ±2·2	
	Angiotensin 5×10^{-7} M	(11)	25·5 ±2·1[b]	+57
Rat vas deferens	Control	(6)	2·85±0·19	
	Angiotensin 10^{-8} M	(3)	4·38±0·54[b]	+54

n*, Indicates the number of individual tissues analysed for formation of catecholamines. a, Signifies $P<0{\cdot}01$ when compared with untreated controls using Student's t test. b, Signifies $P<0{\cdot}05$ when compared with untreated controls using Student's t test.

deferens and guinea-pig atria. Most detailed studies were undertaken with these two tissues. Preliminary observations indicated that the ability of the guinea-pig atria to form CA deteriorated rapidly if the tissues were allowed to stand too long before starting the incubation. For example, if the tissues were kept at 37° C in oxygenated Krebs-Henseleit solution for 1 h before incubation with tyrosine-C^{14}, approximately half the amount of CA was formed in 1 h compared with tissues that were incubated within 10 min of dissection (Table 2). Chilling the tissues to 4° C in oxygenated Krebs-Henseleit did not improve the preservation of the tissue CA biosynthesis. Instead the low temperature severely impaired the subsequent uptake of tyrosine into the atria and possibly, as a consequence of this, also lowered the CA synthesis. These observations prompted us to allow only a minimum time to elapse between dissection and incubation of tissues (not more than 20 minutes). In the intervening period the tissues were kept in oxygenated Krebs-Henseleit bicarbonate solution at 37° C. Similar problems were not encountered with rat vasa deferentia and these tissues were simply kept in Krebs-Henseleit medium at room temperature until the dissection was completed.

TABLE 2. *Effect of preincubation at 37° C or 4° C in Krebs-Henseleit bicarbonate on synthesis of ^{14}C-catechols from ^{14}C-tyrosine in guinea-pig atria*

Treatment	Incubation time (h)	n*	^{14}C-catechols ((dpm)/g)$\times 10^{-3}$ ±S.E.M.	^{14}C-tyrosine uptake ((dpm)/g)$\times 10^{-3}$ ±S.E.M.	Endogenous NA µg/g±S.E.M.
	1	3	43·6± 5·7	1,067±36	—
Preincubation at 37° for 1 h	1	3	25·5± 1·5	939±96	—
	0·5	3	44·6± 1·5	1,166±39·9	3·38±0·21
Preincubated 1·5 h at 4° C	0·5	3	15·3± 1·1	513±61·2	2·60±0·30
	1	3	52·5±13	1,236±24	2·74±0·56
Preincubated 1·5 h at 4° C	1	3	25·8± 0·6	727±48	2·01±0·09

n* Indicates the number of individual tissues analysed for formation of catechols, endogenous NA and ^{14}C-tyrosine content.

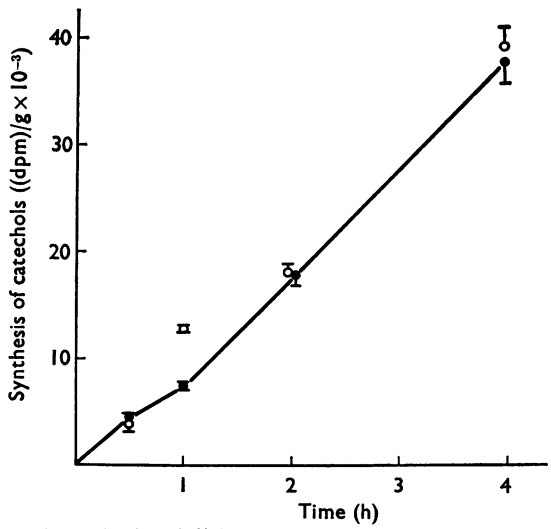

FIG. 1. Time course of synthesis of ^{14}C-catechols from ^{14}C-tyrosine in rat vasa deferentia. Solid circles, controls; open circles, angiotensin 10^{-8}M. Each point is the mean±S.E.M. of values obtained from individual vasa deferentia.

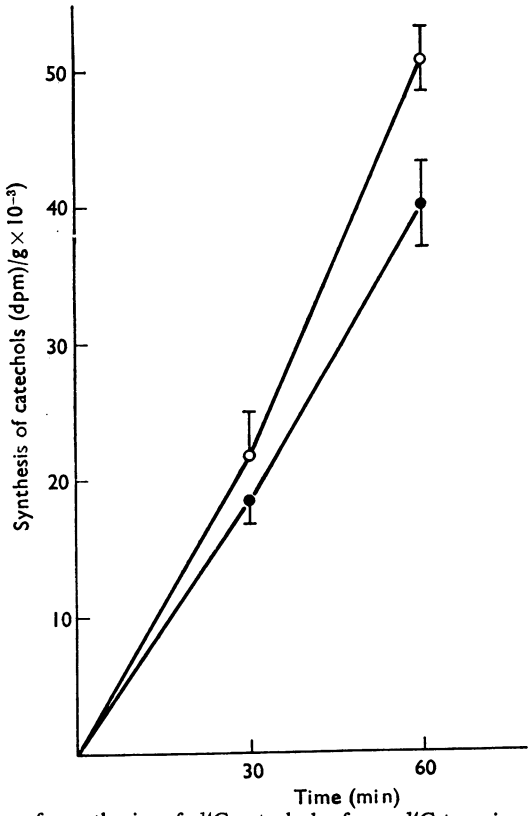

FIG. 2. Time course of synthesis of ^{14}C-catechols from ^{14}C-tyrosine in guinea-pig atria. Solid circles, controls; open circles, angiotensin 5×10^{-8}M. Each point is the mean ± S.E.M. of values obtained from individual atria.

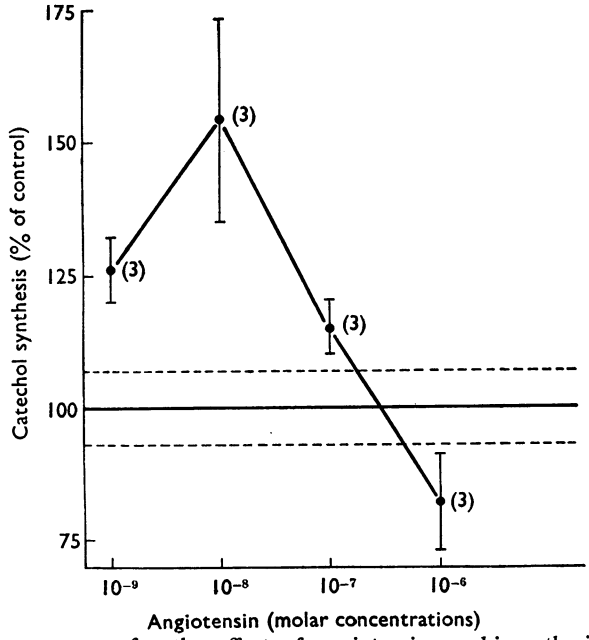

FIG. 3. Dose-response curve for the effect of angiotensin on biosynthesis of ^{14}C-catechols from ^{14}C-tyrosine in rat vasa deferentia. Vasa deferentia were incubated for 1 h at 37° C in a Krebs-Henseleit medium containing ^{14}C-tyrosine and angiotensin-II-amide. Results are expressed as mean % of control catechol synthesis ± S.E.M. Figures in parentheses indicate the number of individual vasa deferentia analysed.

Time course of synthesis

The time course of synthesis of CA from tyrosine-C^{14} was followed in rat vasa deferentia (Fig. 1) and guinea-pig atria (Fig. 2). In both tissues a significant acceleration of CA synthesis in the presence of angiotensin was observed after 1 hour. When rat vasa deferentia were incubated for longer times the results showed clearly that the acceleration of synthesis was not maintained. The synthesis in control tissues was linear throughout this time.

Dose-response curve for angiotensin

The effect of a range of doses of angiotensin-II-amide on the production of labelled CA from tyrosine-C^{14} in rat vasa deferentia during the course of 1 h is shown in Figure 3. There was a significant increase in the synthesis of labelled CA at concentrations of angiotensin as low as 10^{-9}M but the maximal effect with this preparation was seen only at the higher dose of 10^{-8}M. In the presence of still higher concentrations of the polypeptide the synthesis of CA no longer differed significantly from controls.

A dose-response curve was also obtained for guinea-pig atria (Fig. 4); here the maximum effect on CA synthesis was observed in the presence of a concentration of angiotensin of 5×10^{-7}M. At a high concentration of 1×10^{-5}M synthesis of CA fell below that of controls. In addition to enhancing the formation of NA from tyrosine, angiotensin also produced a significant increase in the specific activity of the NA isolated from the atria at the end of the incubation (Table 3).

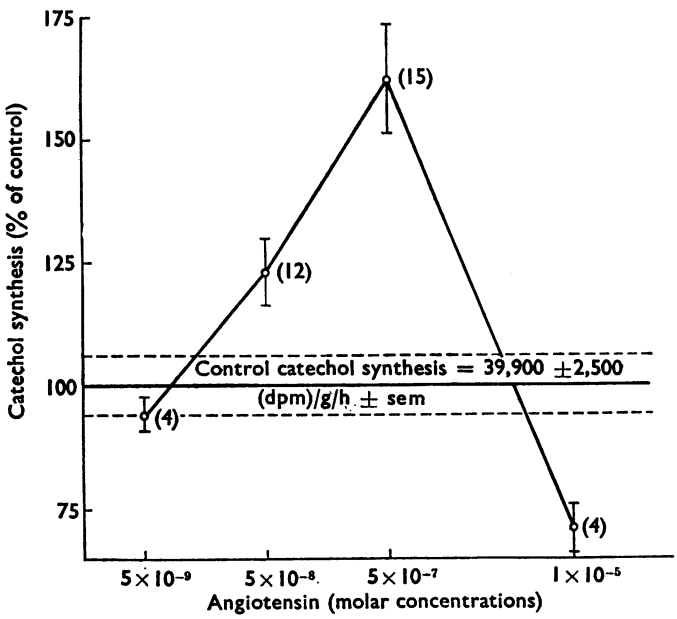

FIG. 4. Dose-response curve for the effect of angiotensin on biosynthesis of ^{14}C-catechols from ^{14}C-tyrosine in guinea-pig atria. Atria were incubated for 1 h at 37° C in a Krebs-Henseleit medium containing ^{14}C-tyrosine and angiotensin-II-amide. Results are expressed as mean % of control catechol synthesis ± S.E.M. Figures in parentheses indicate the number of individual guinea-pig atria analysed.

The pattern of catechol metabolites isolated from control and angiotensin-treated guinea-pig atria 0·5 h and 1 h after the start of the incubation was similar. At 1 h NA accounted for more than 75% of the labelled catechol metabolites in both control and angiotensin-treated tissues (Table 4).

Noradrenaline synthesis from DL-*DOPA*

When the rate-limiting step in the synthesis of NA, tyrosine hydroxylation, was bypassed by using DOPA as the initial substrate instead of tyrosine, the stimulatory effect of angiotensin on NA synthesis in guinea-pig atria was found to disappear entirely.

Effect of angiotensin on release of exogenous and newly synthesized catecholamine

In two experiments with angiotensin concentrations of 5×10^{-9}M and 5×10^{-7}M, the amount of ^{14}C-catechol compounds present in the bath fluids of angiotensin-treated tissues at the end of a 1 h incubation exceeded that of control media by 35% and 88%, respectively. However, in four other experiments carried out with a concentration of angiotensin of 5×10^{-7}M no significant difference was found between the amount of labelled catechols recovered from the incubation media of the control tissues and that of the angiotensin-treated tissues. Angiotensin failed to increase the basal release of newly synthesized ^{3}H-NA from a single pair of guinea-pig atria over 5 min incubation periods. Likewise, in the superfusion experiments the release of exogenously supplied ^{3}H-NA from guinea-pig atria was not changed significantly in the presence of angiotensin (5×10^{-7}M).

TABLE 3. *Specific activity of noradrenaline (NA) isolated from guinea-pig atria after incubation for* 1 *h with* ^{14}*C-tyrosine in the presence and absence of angiotensin*

Treatment	*n*	^{14}C-catechols ((dpm)/g) $\times 10^{-3}$	Endogenous NA μg/g	Specific activity* (dpm)/μg NA
Control	7	26·5±3·7	2·68±0·13	7,368± 812
Angiotensin (5×10^{-8} M)	7	31·8±3·4	2·55±0·19	10,463±1073
Angiotensin (5×10^{-7} M)	7	47·3±5·0[a]	2·80±0·3	14,794±1760[a]

a, Signifies $P<0·01$ when compared with untreated controls using Student's *t* test. *n*, Indicates the number of individual tissues analysed for ^{14}C-catechols and endogenous NA content. *, Corrected for % NA in ^{14}C-catechols (table 4).

TABLE 4. *Distribution of newly formed catechols in control and angiotensin treated guinea-pig atria after incubation with* ^{14}*C-tyrosine*

Incubation time (h)	*n*	Treatment	% Change catechols	Chromatography % NA	% DA	% Deaminated metabolites
0·5	4	Control	—	66·1	10·2	23·7
	4	5×10^{-8}M	+17	66·8	11·8	21·4
1·0	4	Control	—	75·5	5·9	18·6
	4	5×10^{-8}M	+27	82·1	4·5	13·4

n=Indicates the number of experiments.

Inactivation of angiotensin in vitro

There was no loss of biological activity when angiotensin was kept at 37° C in Krebs solution for 1 hour. However, there was a considerable loss of activity when whole guinea-pig atria or vasa deferentia, or rat atria, were included in the incubating solution with the angiotensin. Incubation with 400–600 mg of tissue in 5 ml of Krebs solution containing 250 ng of angiotensin (5×10^{-8}M) resulted in a total loss of biological activity in the rat colon assay.

In further experiments with smaller amounts of tissue it was possible to estimate that 1 g of guinea-pig vas deferens inactivated between 400–600 ng of angiotensin in 30 minutes. In our experiments where CA synthesis was followed, the tissue weights per 5 ml of Krebs were in the range 200–600 mg. It is thus apparent that there was a rapid loss of angiotensin activity under the conditions of our experiments where synthesis was determined over a 1 h period.

Discussion

It is now widely believed that the increase in synthesis of NA observed in sympathetically-innervated tissues which have been stimulated electrically or depolarized by K^+ ions arises because in these situations the rate-limiting enzyme in NA biosynthesis, tyrosine hydroxylase, is freed from end-product inhibition by the release of a small pool of NA from within the neurone (see Weiner, 1970; Boadle-Biber *et al.*, 1970). The possibility that the acceleration of CA synthesis seen in the presence of angiotensin arises in an analogous manner is an attractive one but seems unlikely since in most of the experiments reported here the increase in synthesis of CA induced by angiotensin during incubation for 1 h was not accompanied by any increase in the release of CA into the bath. Furthermore, angiotensin failed to alter the release of both newly synthesized and exogenously supplied NA from tissues examined over much shorter intervals of time. For example, superfusion of guinea-pig atria with a concentration of angiotensin that produces maximal acceleration of CA synthesis (5×10^{-7}M) neither enhanced the basal release of exogenously supplied ^{3}H-NA nor produced any sympathomimetic effects. In superfusion experiments with rabbit portal veins, described elsewhere (Hughes & Roth, 1971) concentrations of angiotensin-II-amide from 10^{-8} to 4×10^{-7}M had no significant effect on the basal efflux of exogenous [^{3}H]-NA. Higher concentrations ($0{\cdot}5–2 \times 10^{-6}$M) caused a small and variable increase in [^{3}H]-NA efflux but this effect was transient and seen only during the first 1–2 min of angiotensin superfusion. Since angiotensin (10^{-8} to 5×10^{-7}M) produced no sympathomimetic effect with the rabbit portal vein, it is unlikely that angiotensin releases NA at these concentrations in the absence of sympathetic nerve stimulation. The finding of Chevillard and coworkers that angiotensin enhances release of NA newly synthesized from DA in slices of rat heart seems to contradict the observations discussed above but can probably be accounted for by the fact that, in their experiments, the uptake of the labelled precursor (DA) was increased in the presence of angiotensin (Chevillard, Duchene & Alexandre, 1971). A similar increase in the overall uptake of the amino acid precursor, tyrosine by angiotensin in the current experiments was not observed.

Our results suggest that it is unlikely that release of CA plays a role in producing the increase in CA biosynthesis seen in the presence of angiotensin. How-

ever, the exact mechanism involved in producing this increase in the CA biosynthesis remains unknown. Further work is now necessary to determine whether angiotensin modulates NA synthesis *in vivo*. It would be fruitless to compare the angiotensin concentrations used by us *in vitro* to those normally found *in vivo* as a guide to possible physiological relevance. Firstly we have shown that our angiotensin activity rapidly declines *in vitro* and secondly, it is possible that the tissues were already influenced by the presence of angiotensin released *in vivo* before dissection and incubation *in vitro*. It also seems as if the effects on NA synthesis are only apparent *in vitro* after most of the detectable angiotensin activity has disappeared from the Krebs medium. These considerations may help to explain the step dose-response curves for the effect of angiotensin on CA synthesis, and also why the effect is not maintained for more than one hour.

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