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An investigation of the effects of angiotensin on the release of neurohumoral transmitters at motor, adrenergic and cholinergic nerve terminals

I. E. HUGHES

On the sciatic nerve-gastrocnemius-soleus muscle preparation of the cat, angiotensin (1–5 μg/kg, i.v.) potentiated the twitch response to maximal and submaximal stimulation of the sciatic nerve and produced partial reversal of an incomplete tubocurarine blockade. These actions could not be explained in terms of increased acetylcholine release since they were not seen in isolated motor nerve-striated muscle preparations and were probably secondary to the cardiovascular actions of angiotensin. Blockade of conduction in the postganglionic cholinergic nerves in the guinea-pig isolated ileum preparation by cooling or anoxia antagonized the response of this tissue to angiotensin. These procedures left the response to exogenous acetylcholine unchanged though they removed the cholinergic component of the response to angiotensin which is known to be present in this tissue. No evidence of increase in catecholamine output could be found in preparations of guinea-pig and rabbit vasa deferentia or rabbit duodenum responding to submaximal stimulation of their adrenergic nerves. It is concluded that angiotensin has no direct action on the stores of neurohumoral transmitter at motor, postganglionic cholinergic or postganglionic adrenergic nerves and that its known acetylcholine releasing action in the isolated ileum results from stimulation of the ganglia.

EARLY work on angiotensin indicated that the vasopressor actions of this peptide derived from a direct action on smooth muscle. More recent work has shown that release of neurohumoral transmitters may play an important role in the response of tissues to angiotensin. This release may be caused by central, ganglion stimulant, or direct action on the stores of neurohumoral transmitter found in the adrenal gland and at the postganglionic nerve terminal (Bickerton & Buckley, 1961; Robertson & Rubin, 1962; Feldberg & Lewis, 1964; Youmans, Davis & others, 1964; Lewis & Reit, 1965).

In the experiments reported in this paper the actions of angiotensin on the release of neurohumoral transmitters from motor, postganglionic cholinergic and post-ganglionic adrenergic nerves have been examined.

Experimental

Sciatic nerve-gastrocnemius-soleus muscle preparation of the cat. Cats of either sex, weighing from 1–3·6 kg were anaesthetized with chloralose (80 mg/kg i.p.), artificially respired and set up in a Brown-Schuster myograph to record twitch tension developed in the right gastrocnemius-soleus muscles in response to electrical stimulation of the distal end of the sectioned right sciatic nerve. Stimuli were applied through platinum hook electrodes from a Palmer H 44 square wave stimulator and twitch tension was measured isometrically by a Grass displacement transducer (FTO3C). Blood pressure was measured from the left femoral artery by an E. & M. linear core pressure transducer and all recordings were made

From the Department of Pharmacology, School of Medicine, Leeds 2, England, and (present address) the Department of Pharmacology, The University of Western Australia, Nedlands, W.A.

on a Beckman RB Dynograph. Drugs were dissolved in saline (0·9% NaCl), injected into the left femoral vein, and washed in with 0·5 ml of saline. All animals were given 500 units/kg heparin injection B.P. as soon as the operative procedure was complete.

Phrenic nerve-diaphragm preparation. (Mogey, Trevan & Young, 1949). Preparations were suspended in modified Krebs solution (NaCl 6·92, KCl 0·35, $CaCl_2$ 0·21, $MgSO_4.7H_20$ 0·29, $NaHCO_3$ 2·1, KH_2PO_4 0·162, glucose 2·0 g/litre) at 37° and gassed with oxygen 95% carbon dioxide 5%. The response of the muscle to electrical stimulation of the phrenic nerve was recorded by the system described above.

Transmurally stimulated vas deferens and isolated rabbit duodenum preparations. These preparations were prepared as described by Clark & Hughes (1966).

Coaxially stimulated guinea-pig ileum. Short (2–3 cm) pieces of guinea-pig ileum taken from 5 cm above the ileocaecal junction were suspended in modified Tyrode solution (NaCl 8·0, KCl 0·2, $CaCl_2$ 0·15, $MgCl_2{\cdot}6H_2O$ 0·2, $NaHCO_3$ 1·0, $NaH_2PO_4{\cdot}2H_2O$ 0·05 glucose 2·0 g/litre) at 34° and gassed with oxygen 95%, carbon dioxide 5%. Coaxial stimulation was applied as described by Paton (1955) and longitudinal contractions were recorded isotonically on smoked paper by a frontal writing lever (load 1–2 g).

Anoxia was induced by replacing the oxygenated Tyrode solution by Tyrode solution made in freshly boiled distilled water which had been cooled under nitrogen. The arrangement of heating coils in the bath was such that the change-over from normal Tyrode to oxygen-free Tyrode solution could be accomplished by turning a three-way tap. Thus no break in dose schedule or alteration in temperature took place at the change-over. During the period of anoxia the bath was gassed with nitrogen 95%, carbon dioxide 5%. In all cases washing of the tissue was performed by overflow and agonists were added to the bath by pipette; in no case did the dose volume exceed 2% of the total bath volume.

The following drugs were used: acetylcholine chloride, atropine sulphate monohydrate, cinchocaine hydrochloride, heparin injection B.P., lignocaine hydrochloride monohydrate, morphine sulphate, procaine hydrochloride, (+)-tubocurarine chloride, val-5-hypertensin II asp-β-amide (Hypertensin, Ciba). All doses are expressed in terms of these salts.

No deterioration of dilute solutions of angiotensin took place over the period of the experiment when the solutions were stored in plastic vessels or in glass flasks treated with a silicone preparation.

Results

Sciatic nerve-gastrocnemius-soleus preparation of the cat. Injections of angiotensin (1·5 μg/kg) produced the expected rise in mean arterial pressure and potentiated the response of the gastrocnemius-soleus muscles to electrical stimulation of the sciatic nerve. Increase in twitch tension

was invariably observed in each of 5 preparations and occurred whether nerve stimulation was maximal or submaximal (Fig. 1a). This increase in twitch tension induced by angiotensin could be demonstrated repeatedly in the same preparation. Some degree of reversal of a partial tubocurarine blockade could also be demonstrated though this was not as marked as that caused by tetraethylammonium (TEA) (Fig. 1b). The angiotensin induced potentiation of the twitch response was of short duration and appeared related to the duration of the pressor response. The increase in twitch tension was however delayed by at least 40 sec after the blood pressure started to rise.

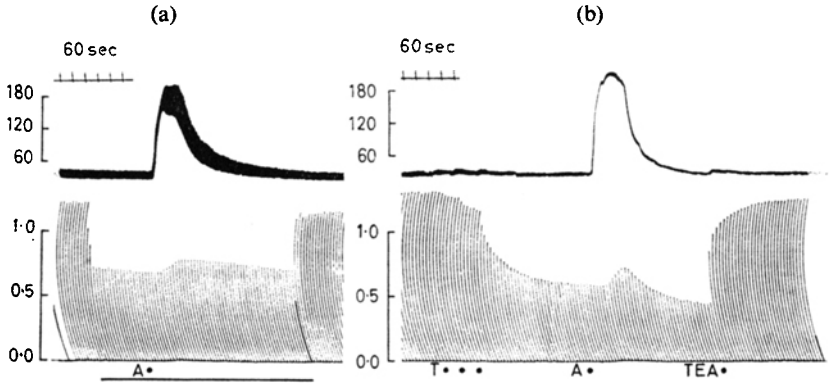

FIG. 1 (a). Cat, 2·05 kg, (b) cat 2·15 kg, under chloralose anaesthesia (80 mg/kg, i.p.). Upper record—femoral arterial blood pressure. Lower record—twitch tension developed in the gastrocnemius-soleus muscles in response to stimulation of the sciatic nerve at a rate of 3/min at 40 V 500 μsec duration in (a) and (b), and also submaximally in (a) at 2·8 V for 30 μsec duration applied as indicated by the black line. Angiotensin (A) (5 μg/kg, i.v.) was administered at the black dot in (a). In (b) its effect at the same dose with tetraethylammonium bromide (5 mg/kg, i.v. at TEA) on a partial tubocurarine blockade produced by 3 × 0·25 mg/kg tubocurarine (i.v.) at T.

Phrenic nerve-diaphragm preparations. In concentrations up to 5×10^{-6} g/ml, angiotensin showed no effect on the maximally or submaximally stimulated rat phrenic nerve-diaphragm preparation (Fig. 2a). Neither was any effect observed on the rate of onset (Fig. 2b) or rate of recovery from a partial tubocurarine blockade. TEA however produced a marked reversal of the partial tubocurarine blockade whether administered during the onset or recovery phases of the block.

In a single experiment on a phrenic nerve-diaphragm preparation taken from a cat, angiotensin produced no potentiation of the response to submaximal nervous stimulation in concentrations up to 1×10^{-6} g/ml.

Coaxially stimulated guinea-pig ileum. Various procedures were tried in an attempt to block the postganglionic nerve fibres in the ileum without affecting the response of the tissue to exogenous acetylcholine. The purpose was to discover whether the release of acetylcholine, known to be produced by angiotensin, results from a direct action of angiotensin on the nerve terminals or from the ganglion stimulant actions of angiotensin.

(*a*) *Effect of local anaesthetics.* Concentrations of procaine, lignocaine and cinchocaine which were just sufficient to block the response of the tissue to coaxial stimulation were found to antagonize the response to exogenous acetylcholine.

(*b*) *Effect of cooling.* Cooling is known to produce a failure of conduction in nerve fibres and was therefore used as an alternative procedure to block postganglionic nerve conduction. A maintained reduction in the temperature of the bath to 10° abolished the response to coaxial stimulation within 30 min. The response of the tissue to exogenous acetylcholine

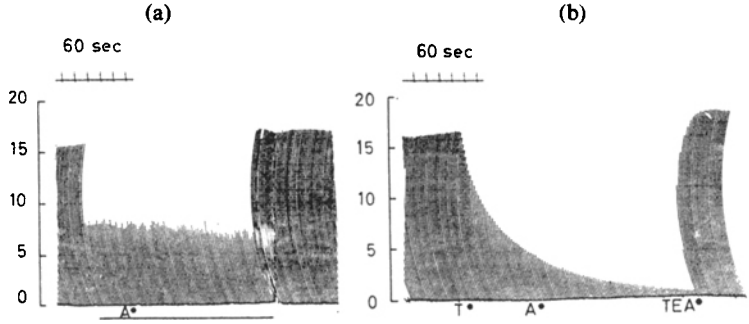

FIG. 2 (a) and (b). Rat phrenic nerve—diaphragm preparation suspended in Krebs solution at 37° responding to supramaximal stimulation (10 V, 200 μsec duration) of the phrenic nerve at a rate of 6/min in (a) and (b), also in (a) to submaximal stimulation (8 V, 30 μsec duration) applied as indicated by the black line. Angiotensin was administered to a final concentration of 5×10^{-6} g/ml at A. Its effect and that of tetraethylammonium bromide (1×10^{-4} g/ml at TEA) on a partial competitive neuromuscular blockade produced by a concentration of tubocurarine of 2×10^{-6} g/ml (at T) is shown in (b).

was unaffected by this procedure. When previously effective doses of angiotensin were administered to the cooled tissue no response was observed while angiotensin was in contact with the tissue. As soon as washout started however, a strong slow progressive contraction developed whether the angiotensin had been in contact with the tissue for a short or a long time (1 or 5 min). The contraction developing on washout lasted for several min despite further washing of the tissue.

(*c*) *Effect of anoxia.* Complete abolition of the response to coaxial stimulation was quickly achieved on supplying the tissue with oxygen-free Tyrode solution. Anoxia did not greatly affect the response of the tissue either to exogenous acetylcholine or to histamine but the response to angiotensin was markedly reduced. Ratios for equi-active doses of angiotensin before and during the period of anoxia were between 50 and 100 (6 experiments) although in one experiment the dose ratio was over 200 (Fig. 3). Response to coaxial stimulation returned to the control value in approximately 45 min after the oxygen supply was restored and tissue responses to histamine, acetylcholine and angiotensin had also returned to their control values after this time. Atropine (1×10^{-9} g/ml) was effective in antagonizing the response to angiotensin in experiments

not involving anoxia but failed to do so in each of two experiments where its action was tested during the period of anoxia.

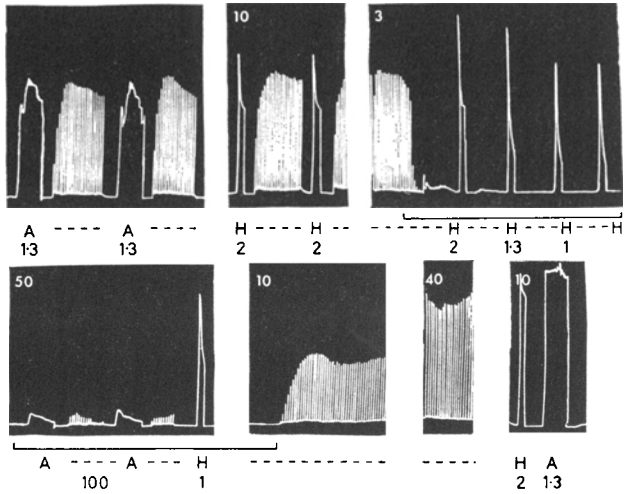

FIG. 3. Guinea-pig ileum preparation suspended in Tyrode solution at 34°. Showing the effect of anoxia (solid bars) on the response of the tissue to histamine (H), angiotensin (A) and coaxial stimulation (50 V, 1 msec duration, 6/min indicated by the broken bars). The numbers at the top of the records indicate the time (min) elapsed between the records. All concentrations are expressed in terms of μg/ml final bath concentration.

Transmurally stimulated vas deferens. In 6 experiments angiotensin, in concentrations up to 1×10^{-6} g/ml, had no effect on the response of guinea-pig or rabbit vasa deferentia to maximal or submaximal electrical stimulation (Fig. 4).

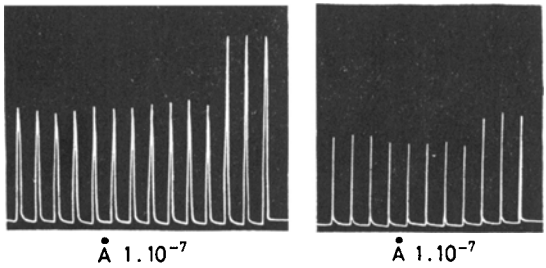

FIG. 4. Vas deferens preparation from the guinea-pig (left) and rabbit (right) suspended in Tyrode solution at 34° and responding to submaximal transmural stimulation (100 V, 400 μsec duration, 20/sec for 20 sec in every 5 min). For the last three responses on each record the repetition rate was raised to 50/sec angiotensin (Ang. 1×10^{-7} g/ml) was added to the bath as shown.

Isolated rabbit duodenum. The spontaneous pendular movements exhibited by this preparation are inhibited by stimulation of the periarterial nerves (Finkleman, 1930). Small doses of angiotensin (2–7·5

$\times 10^{-9}$ g/ml) caused a slight increase in the tone of the preparation but had little effect on the size of the spontaneous movements. These small doses of angiotensin did not influence the degree of inhibition of the pendular movements caused by submaximal stimulation of the periarterial nerves. In one experiment a slight decrease in the size of the inhibition was noted but on no occasion was an increase found (Fig. 5).

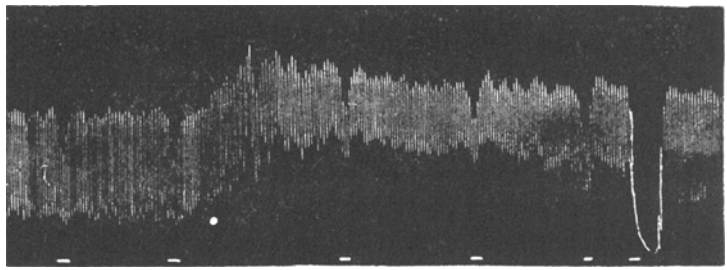

FIG. 5. Rabbit duodenum suspended in Tyrode solution at 36°. Showing the effect of angiotensin ($7{\cdot}5 \times 10^{-9}$ g/ml at A) on the response to submaximal stimulation of the periarterial nerves (40 V, 300 μsec duration, 10/sec for 20 sec) as indicated by the white bars. The repetition rate was raised to 50/sec before the last response.

Discussion

It has been established (Khairallah & Page, 1961: Robertson & Rubin, 1962) that acetylcholine contributes appreciably to the response of isolated rabbit and guinea-pig ilea to angiotensin. This release of acetylcholine by angiotensin could be the result of a direct action of angiotensin on the stores of acetylcholine at the postganglionic cholinergic nerve terminal. If this were so then it might be expected that a similar release might be shown at the neuromuscular junction. The potentiation of the twitch response of the gastrocnemius-soleus muscles to maximal and submaximal electrical stimulation of the sciatic nerve and the partial reversal of the competitive neuromuscular blockade which were induced by angiotensin could be accounted for in terms of an increased release of acetylcholine. If this explanation were correct then angiotensin would be expected to show a similar effect on isolated motor nerve-striated muscle preparations such as the rat phrenic nerve-diaphragm. However, angiotensin did not potentiate the effect of stimulation of the phrenic nerve in this isolated preparation. This difference in the *in vivo* and *in vitro* responses is unlikely to be due to a species variation as observations made on a single phrenic nerve-diaphragm preparation taken from a cat accorded with those in the rat.

It seems more likely that the potentiation of the effects of electrical stimulation of the sciatic nerve seen in the cat is due to a secondary effect of the action of angiotensin on the cardiovascular system. The large pressor response produced by angiotensin might be expected to change both pressure and flow in the limb. These changes in flow will affect

oxygenation and other factors in the tissue and may be responsible for the potentiation of the effects of stimulation of the sciatic nerve. In addition, angiotensin is known to release catecholamines from the adrenal gland (Felberg & Lewis, 1964: Robinson, 1965) and this is especially likely to occur with the high doses used in these experiments. It is well known that catecholamines are capable of facilitating the process of neuromuscular transmission (Bowman & Zaimis, 1958: Krnjević & Miledi, 1958) and the release of catecholamines may well contribute to the potentiation of the twitch response seen in these experiments. The delayed onset of the potentiation of the twitch response as compared with the pressor response may be due to the time required for angiotensin to release catecholamines from the adrenal gland into the general circulation and hence to the gastrocnemius and soleus muscles.

As no increase in the release of acetylcholine at the neuromuscular junction could be demonstrated, attempts were made to discover if the presence of ganglia and of functional postganglionic nerves was necessary for angiotensin to induce a release of acetylcholine in the guinea-pig ileum.

Blockade of conduction in postganglionic nerves by local anaesthetics was found to be accompanied by atropine-like actions. Local anaesthetics could not be used therefore to distinguish between acetylcholine released by a direct action on the store and acetylcholine released by nerve impulses induced in the postganglionic nerves.

Although the experiments involving cooling of the ileum were complicated by a washout phenomenon the cold preparation showed a great reduction in the response to angiotensin but not to acetylcholine. This reduction in the response was probably due to a blockade of conduction in the postganglionic nerve fibres which eliminated the ganglionic component of the response to angiotensin. Any release of acetylcholine produced by a direct action of angiotensin on the stores of acetylcholine would be unaffected by this procedure since the cold tissue responded well to exogenous acetylcholine. However, the release processes may have been affected by cooling.

The antagonism of the response to angiotensin produced by anoxia is probably also due to failure of conduction in the post-ganglionic nerves and the elimination of the ganglionic component of the response. The failure of atropine to antagonize the response to angiotensin during the period of anoxia indicates that the cholinergic component of the response has been removed by this treatment. The large ratio between equieffective doses before and during the period of anoxia indicates that, in normal ileum, a large proportion of the response to angiotensin is mediated by acetylcholine.

The suggestion that ganglion stimulation is responsible for the release of acetylcholine by angiotensin is supported by the findings of Godfraind, Kaba & Polster (1966). They found that there was no cholinergic component in the response of isolated strips of longitudinal muscle taken from guinea-pig gut. This preparation is known to contain postganglionic cholinergic nerves since electrical stimulation produces a response that is

completely abolished by lachesine (Rang, 1964). The absence of ganglia in the longitudinal muscle strip probably accounts for the lack of any cholinergically mediated action of angiotensin in this preparation.

The failure of hexamethonium and related compounds to antagonize the response of the guinea-pig ileum to angiotensin is not in conflict with this hypothesis. It has been shown in isolated ganglia that the ganglion stimulant action of angiotensin is insensitive to hexamethonium but is sensitive to morphine (Lewis & Reit, 1965, 1966; Trendelenburg, 1966). This drug is an effective antagonist of angiotensin on the guinea-pig ileum though at least some of the action will be due to the inhibition of the release of acetylcholine from the postganglionic nerve terminal which is produced by morphine (Schaumann, 1957).

No evidence was found that angiotensin increased catecholamine release when tested on preparations responding to electrical stimulation of adrenergic nerves. Thus angiotensin produced no increase in the tension developed by guinea-pig or rabbit vasa deferentia in response to maximal or submaximal transmural stimulation. Similarly, angiotensin produced no increase in the degree of inhibition produced by standard submaximal stimulation of the periarterial nerves to the rabbit duodenum preparation.

These results are in accord with the findings of Hertting & Suko (1966) who could find no increase in the output of tritiated noradrenaline in response to stimulation of nerve fibres in the presence and absence of angiotensin. The results obtained on the guinea-pig vas deferens preparation are however in contrast to those obtained by Benelli, Della Bella & Gandini (1964) who found that angiotensin produced a potentiation of the response of the guinea-pig vas deferens to submaximal stimulation of the hypogastric nerve. However, the preparation used by Benelli and his co-workers contains ganglia (Birmingham & Wilson, 1963) and this may account for the potentiation. In addition, unpublished experiments have revealed a variation in the response of the vas deferens to angiotensin depending on the ionic composition and temperature of the bathing fluid.

CONCLUSION

The experimental evidence presented supports the view that angiotensin has no direct effect on the stores of neurohumoral transmitter at the endings of motor, postganglionic cholinergic or postganglionic adrenergic nerves and that the release of transmitter which mediates the response of some tissues to angiotensin is a result of the known ganglion stimulant action of angiotensin.

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