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STIMULATION OF INTESTINAL NERVOUS ELEMENTS BY ANGIOTENSIN

BY

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The stimulation of nervous elements in the intestine by the polypeptide angiotensin has been examined. Comparisons between nicotine, a neurotropic agent, acetylcholine which exerts principally a musculotropic action and angiotensin have been made by examining their interactions with hexamethonium and the anticholinesterase 1,5-di-(*p*-*N*-allyl-*N*-methylaminophenyl)pentan-3-one dibromide in both the rabbit and guinea-pig ileum. The effect of atropine on the responses of the guinea-pig ileum and botulinum toxin on the responses of the rabbit ileum to angiotensin was also tested. The results show that angiotensin causes most of its contractile response in both the guinea-pig and rabbit ileum by an indirect action, through the stimulation of nervous tissue.

The vasopressor action of angiotensin has been assumed to be due to a direct musculotropic effect on the vascular smooth muscle. This conclusion has been drawn from a number of studies in which pharmacological blocking agents did not apparently affect the vascular responses to angiotensin.

On the basis of the above assumption we attempted to use a semi-purified angiotensin preparation as a purely direct smooth muscle stimulant for control purposes in experiments with anticholinesterases on intestinal preparations. However, it was found that the angiotensin contractions were potentiated by anticholinesterases. Consequently the investigation was extended to determine the exact mode of action of angiotensin, using a more highly purified preparation, on isolated intestinal segments. The analysis by means of botulinum toxin of drug responses due to an indirect action on the nervous elements of the intestine has been outlined by Ambache & Lessin (1955), and by the use of this method we have now obtained evidence for a dual activity on the intestine. The results have been the subject of a previous brief communication (Robertson & Rubin, 1958).

METHODS

Isolated intestine. Rabbits and guinea-pigs were killed by a blow on the back of the neck. 3 to 4 cm segments of ileum, taken 25 to 30 cm below the pylorus, were suspended in a 10 ml. isolated organ-bath of Mg^{++}-free Tyrode solution containing 1.1 g/l. sodium bicarbonate and aerated with 95% O_2 and 5% CO_2 for rabbit ilea or for guinea-pig ilea 1.0 g/l. sodium bicarbonate aerated with 100% O_2. Contractions of the longitudinal muscle were recorded by means of a lightly laden, isotonic frontal writing lever.

Acetylcholine and nicotine were added to the bath for 30-sec periods every 90 sec in the case of guinea-pig ilea and every 120 sec for rabbit ilea. Angiotensin was left in contact

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with the tissue for periods of up to 4 min in both species. The anticholinesterase 1,5-di-(*p*-*N*-allyl-*N*-methylaminophenyl)pentan-3-one dibromide (BW 284C51), atropine or hexamethonium were added to the bathing fluid contained in a separate reservoir which was connected to the organ bath via a 2-way tap so that an intermediate interchange between this solution and normal bathing fluid could be readily effected.

Contraction heights of the rabbit ileum were measured from the mid-point of the preceding pendulum movements to the mid-point of the pendulum movements occurring during the contraction (Ambache & Lessin, 1955).

Experiments with botulinum toxin. Immediately before use the toxin was dissolved in a minimal amount of buffer solution consisting of 1% disodium phosphate and 0.2% gelatin, acidified to *p*H 6.95 with hydrochloric acid. The toxin was used as follows:

When control responses to stimulating drugs were established the volume of the bathing fluid was reduced to 2 to 3 ml. and then the tissue left in contact for 30 min at 37° C with 10 to 20 mg of toxin type A (equivalent to 2.5 to 5×10^7 mouse LD50) or for 60 min with 100 mg type D (equivalent to 3×10^6 mouse LD50). At the end of the contact period the toxin was removed from the bath and inactivated in hypochlorite solution. The organ bath was washed and refilled with Mg^{++}-free Tyrode solution, and after a rest period of 10 to 20 min drugs were reapplied to the tissue. Initially a weaker preparation of botulinum toxin type D, containing 5×10^5 mouse LD50 per mg, was tried but found completely ineffective after contact with the tissue for 1 hr. As in these initial experiments there was little if any reduction in responses to any stimulant drugs, and pendulum movements were relatively unchanged, it was assumed that the treatment with phosphate gelatin in Tyrode solution did not invalidate any results obtained with botulinum toxin.

Drugs. Doses of drugs are expressed in terms of their respective salts: nicotine acid tartrate, acetylcholine chloride, hexamethonium iodide, atropine sulphate and histamine acid phosphate.

Initially a solution of angiotensin I of 10 u./ml. was used. Later experiments were done with a highly purified solid mixture of angiotensin I and II and the earlier experiments repeated. The doses of angiotensin in the text refer to the latter purified mixture of both types I and II, and are expressed in Indianapolis units.

RESULTS

Rabbit ileum. In rabbit isolated intestinal segments angiotensin (0.1 to 0.6 u./ml.) initiated slow contractions which reached their maximum height within 3 min. Tachyphylaxis was not apparent when intervals of at least 8 min were allowed between successive applications of doses.

Interaction with an anticholinesterase. BW 284C51 is a specific inhibitor of true cholinesterase (Copp, 1953; Austin & Berry, 1953; Fulton & Mogey, 1954) and has previously been successfully used in this capacity on the isolated rabbit ileum (Robertson, 1954; Hobbiger, 1954; Ambache & Lessin, 1955). In the present experiments, after constant control responses to at least 2 different doses of stimulating drugs had been obtained, BW 284C51 was added to the bathing fluid to produce a final concentration of 2.5×10^{-7} g/ml. An immediate contraction occurred and this was followed by relaxation which was complete within approximately 10 min. Then the interaction of angiotensin with the anticholinesterase was compared with those of acetylcholine and nicotine (Fig. 1). In all of 4 experiments the responses to all three stimulants were reversibly potentiated; responses to nicotine (5×10^{-7} to 10^{-6}) were increased to a greater extent than those to angiotensin (0.6 u./ml.) whilst those to acetylcholine (1 to 4×10^{-8}) were the least affected.

These results implied that at least part of the action of angiotensin in this tissue was mediated by the release of acetylcholine. However, the possibility existed that the potentiation of the angiotensin response was due to a sub-threshold sensitization produced by the anticholinesterase. This possibility was investigated by examining the interactions of BW 284C51 with a direct-acting stimulant which was not inactivated by cholinesterase. As there was not available a purely direct-acting cholinergic stimulant, carbachol in the presence of the ganglion-blocking agent hexa-

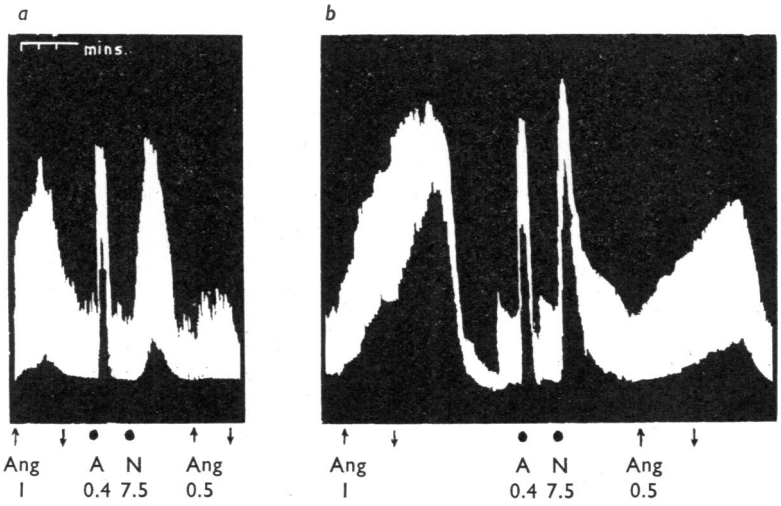

Fig. 1. Responses of the rabbit ileum to the effect of the anticholinesterase BW 284C51 (BW) in potentiating acetylcholine (μg/10 ml.) (A), nicotine (μg/10 ml.) (N) and angiotensin (Indianapolis units) (Ang). Arrows indicate addition and removal of angiotensin from the organ bath alone at (*a*) and in the presence of the anticholinesterase BW 284C51 2.5×10^{-7} at (*b*). BW 284C51 perfusion was begun approximately 15 min before (*b*).

methonium was used. In all of 4 experiments the responses to carbachol (2 to 8×10^{-8}) were reversibly decreased by approximately 10% by BW 284C51 (2.5×10^{-7}).

The latter result confirms those of Ambache & Lessin (1955), who found that BW 284C51 exerted a slight atropine-like effect on rabbit ileum. Thus it appears unlikely that this anticholinesterase produces a direct sensitization of the tissue, and it is concluded that its potentiation of the angiotensin response is due to its anticholinesterase activity.

Interaction with hexamethonium. In the early experiments previously reported (Robertson & Rubin, 1958) hexamethonium was found to cause some reduction in the angiotensin response on the rabbit ileum. However, in later experiments using the more highly purified preparation of angiotensin I and II, we were unable to confirm the earlier findings and found no change in angiotensin responses after hexamethonium.

Interaction with botulinum toxin. Botulinum toxin provides a convenient means of "denervating" the cholinergic supply to the ileum, thus abolishing the parasympathomimetic action of neurotropic substances (Ambache, 1954, 1955). Approxi-

mately equivalent responses to acetylcholine, nicotine and angiotensin were obtained on fresh preparations. Botulinum toxin was left in contact with the tissue (as described under Methods) and the responses to the original doses of the stimulating substances were re-examined following the removal of the toxin. Whereas the responses to acetylcholine were virtually unaltered in all of 4 experiments, those to nicotine were completely abolished whilst those to angiotensin were abolished or markedly reduced.

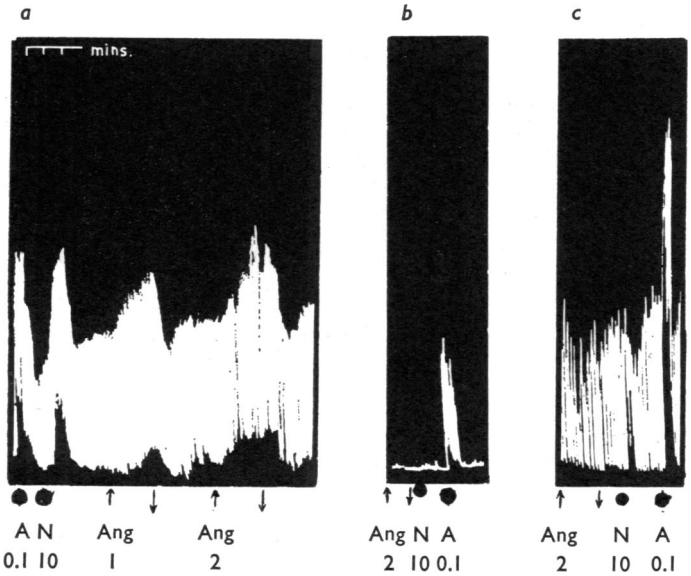

Fig. 2. Responses of rabbit ileum to acetylcholine (μg/10 ml.) (A), nicotine (μg/10 ml.) (N) and angiotensin (Ang) (u./10 ml.). Arrows indicate the addition and removal of angiotensin. Botulinum toxin type A 20 mg was added to the bathing fluid between (*a*) and (*b*) and left in contact with the tissue in 2 to 3 ml. Tyrode solution for 30 min. No response to control doses of angiotensin or nicotine occurred although acetylcholine was still active. BW 284C51 was added to the bathing fluid to give a concentration of 2.5×10^{-7} 10 min prior to (*c*), and there was an immediate return of pendulum movements. The response to acetylcholine was potentiated, while nicotine and angiotensin now produced an inhibitory reaction.

Fig. 2 shows this effect, for, although 2 u. angiotensin and 10 μg nicotine no longer initiated contractions 20 min after the removal of the toxin, 0.1 μg acetylcholine was still active. The acetylcholine response may appear at first sight to be diminished, but this is because of the absence of pendulum movements on which the contraction is normally superimposed.

Pendulum movements were often temporarily abolished by toxin treatment but usually reappeared within 2 hr. In the only toxin experiment in which it was used BW 284C51 hastened the return of pendulum movements when administered 1 hr after toxin treatment (as shown in the last section of Fig. 2). As usual the response to acetylcholine was potentiated by the anticholinesterase, but under these conditions both nicotine and angiotensin caused a slight inhibition of pendulum movements.

Guinea-pig ileum. The guinea-pig ileum was found to be more sensitive to angiotensin than the rabbit ileum, stimulant doses for suitable responses being 0.025 to 0.4 u./ml. The response was also quicker in onset in the guinea-pig ileum, the maximum contraction height being attained within 90 sec of contact. Angiotensin was administered with intervals of at least 8 min between successive doses and no tachyphylaxis was observed.

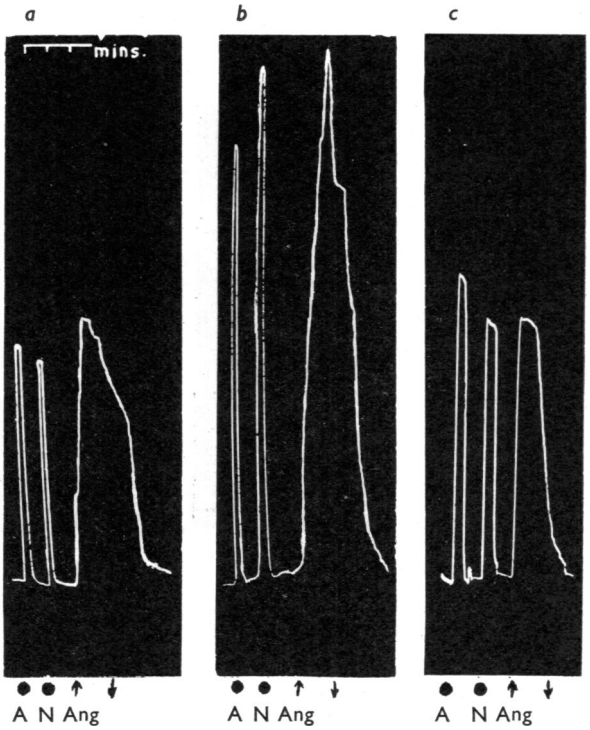

Fig. 3. Responses of the guinea-pig ileum suspended in 10 ml. Tyrode solution to acetylcholine 0.05 μg (A), nicotine 10 μg (N) and angiotensin 0.25 u. (Ang) alone at (*a*) and in the presence of BW 284C51 (2×10^{-9}) at (*b*). Angiotensin was added to the organ bath at ↑ and removed at ↓. The interval between control responses (*a*) and responses shown after the commencement of BW 284C51 perfusion (*b*) was 15 min, and 20 min elapsed before the recovery responses shown at (*c*) were obtained after removal of BW 284C51.

Interaction with an anticholinesterase. The true cholinesterase inhibitor BW 284C51 was used in concentrations of 2×10^{-9} to 10^{-8}. In all of 4 experiments BW 284C51 reversibly potentiated the responses to acetylcholine, angiotensin and nicotine (Fig. 3). As with rabbit ileum the possibility that BW 284C51 might exert a direct sensitizing effect was investigated. However, in the concentration stated above, this anticholinesterase neither potentiated nor depressed the responses to carbachol in the presence of hexamethonium.

Interaction with hexamethonium. Collins (1947, 1948), Ross, Ludden & Stone (1960) and Khairallah & Page (1961) found that angiotensin octapeptide was not antagonized by hexamethonium. In all of 6 experiments in the present investigation concentrations of hexamethonium (2×10^{-5} to 10^{-4}) which completely abolished the

motor responses to nicotine did not diminish those to acetylcholine or to angiotensin. In 3 of these experiments the responses to angiotensin were slightly potentiated in the presence of hexamethonium.

Interaction with atropine. We have confirmed the findings of Khairallah & Page (1961) that atropine antagonizes angiotensin in the guinea-pig ileum. In all of 8 experiments atropine (2×10^{-9} to 10^{-8}) reversibly reduced the responses to angiotensin by 65 to 85% and completely blocked the control responses to acetylcholine (Fig. 4). As is well known, the responses to acetylcholine are inhibited by lower

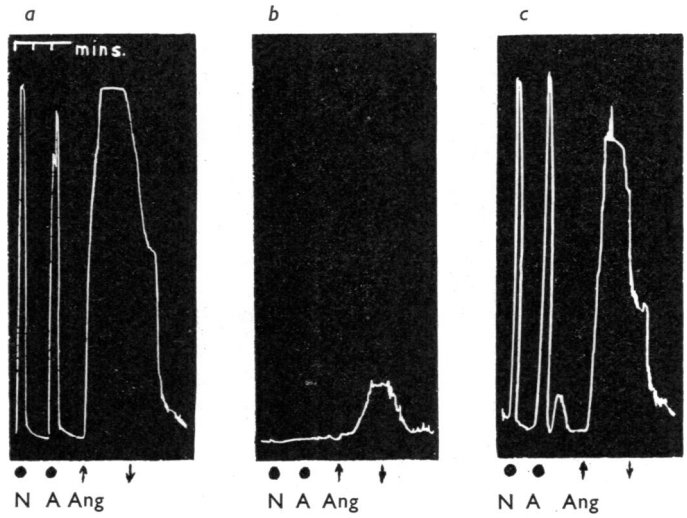

Fig. 4. Responses of guinea-pig ileum suspended in 10 ml. Tyrode solution to nicotine 8 μg (N), acetylcholine 0.08 μg (A) and angiotensin 0.8 u. (Ang) alone at (*a*) and in the presence of atropine (4×10^{-9}) at (*b*). Angiotensin was added to the organ bath at ↑ and removed at ↓. Atropine was added to the perfusion fluid 20 min before the responses shown in (*b*), and removed approximately 40 min before the responses shown on recovery at (*c*).

concentrations of atropine than are those to nicotine. In these experiments angiotensin responses were affected only by the higher concentrations of atropine required to abolish the nicotine responses. The interaction of histamine with atropine was investigated to test whether the concentrations of atropine used affected the responses to non-cholinergic stimulants. Histamine responses were only slightly reduced (by 10 to 15%) in contrast to those of angiotensin, which were markedly reduced (by 65% or more).

DISCUSSION

The present work confirms that BW 284C51 potentiates the responses to acetylcholine and nicotine on isolated intestinal segments (Robertson, 1954) but not those to muscarinic substances unaffected by cholinesterase (Ambache & Lessin, 1955). Thus it can be assumed that BW 284C51 exerts its potentiating effect by its strong and specific anticholinesterase action. In both guinea-pig and rabbit ileum preparations this anticholinesterase potentiated the angiotensin contractions in concentrations which also potentiated the acetylcholine and nicotine responses. Therefore it appears that part of the angiotensin-induced contraction is mediated via a

release of acetylcholine, indicating the stimulation of intestinal nervous elements by angiotensin.

The concentrations of BW 284C51 used in these experiments were lower than those causing substantial inhibition of true cholinesterase activity in homogenized tissue. Koelle (1955) found that a concentration of 3×10^{-5} M was required to inhibit over 95% of the true cholinesterase activity of rabbit brain homogenates, whereas, in the present work, concentrations of approximately 10^{-6} M were used on segments of isolated ileum from the same species. It is possible that a potentiation of cholinergic responses occurs when considerably less than 95% of the enzyme is inhibited or, more probably, in isolated tissues a certain concentration of this anti-cholinesterase produces a greater degree of inhibition of external cholinesterase than it does in homogenates in which both external and internal cholinesterases are available to the inhibitor (McIsaac & Koelle, 1959).

Austin & Berry (1953) found that guinea-pigs were more sensitive to the anti-cholinesterase activity of BW 284C51 than the other species tested by them. This was confirmed in the present work in which concentrations of approximately 10^{-8} M BW 284C51 were used in guinea-pig ileum preparations, that is, approximately 1% of the concentration found necessary in the rabbit. The slight atropine-like action of BW 284C51 reported by Ambache & Lessin (1955) and now seen in the rabbit ileum was not evident in the guinea-pig where the lower doses of the inhibitor were used.

The responses to angiotensin were not reduced by hexamethonium in this series of experiments. The earlier reduction in the responses to angiotensin seen in the rabbit after hexamethonium may thus have been due to impurities. In the guinea-pig some potentiation of the responses to angiotensin occurs after hexamethonium, and this is in agreement with the finding of Collins (1947, 1948) that the ganglion blocking agent tetra-ethyl ammonium potentiates the contractions of the guinea-pig ileum to angiotensin. There are some substances which stimulate smooth muscle indirectly but the responses of which are not reduced by hexamethonium, e.g., 5-hydroxy-tryptamine (Robertson, 1953) and Darmstoff (Ambache & Lessin, 1955). Therefore it is possible that angiotensin may stimulate the ileum partly by indirect means even though its effects are not blocked by hexamethonium.

In the guinea-pig ileum, atropine in low doses inhibits post-ganglionic para-sympathomimetic responses but has very little effect on contractions produced by direct muscle stimulants. Angiotensin responses were markedly reduced by concentrations of atropine which just abolished the responses to nicotine, confirming that part of the action of angiotensin here is indirect.

The most significant results indicating an indirect action of angiotensin were obtained using botulinum toxin on the rabbit ileum after which the responses to angiotensin were reduced or abolished. Botulinum toxin permanently paralyses cholinergic neurones (Ambache, 1949) by blocking the release of acetylcholine from nerve endings (Burgen, Dickens & Zatman, 1949). The use of this toxin to determine whether a smooth muscle stimulant acts directly or indirectly is well established (Ambache & Lessin, 1955).

The slight inhibitory responses to angiotensin and nicotine in the rabbit ileum treated with botulinum toxin seen after the return of the pendulum movements are similar to those of the nicotine reversal as described by Ambache (1951).

The present investigation shows that angiotensin stimulates isolated intestinal smooth muscle both directly and through its nervous elements. Whether part of the effect of angiotensin on blood pressure is also indirect is open to question. The bulk of the evidence to date suggests a strong direct component, but in some studies there are indications that the pressor response to angiotensin is very slightly reduced in the presence of adrenergic neurone blocking drugs (Bumpus, Schwarz & Page, 1957; Bianchi, de Schaepdryver, de Vleeschhouwer & Preziosi, 1960). The results of the present work allow the possibility that part of the pressor effect may be mediated indirectly. Most of the possible sites of such an action have been excluded by previous workers, but not, for example, that of the vasomotor axon reflex described by Hilton (1954).

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