THE INTERACTION OF ANGIOTENSIN II WITH OTHER SPASMOGENS ON VASCULAR SMOOTH MUSCLE

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ABSTRACT

The interaction of angiotensin II with exogenous and endogenous noradrenaline was studied on isolated vascular tissues taken from the rabbit. Angiotensin II potentiated responses to nerve stimulation, but not to noradrenaline, when infused into the isolated ear artery, isolated whole ear and isolated mesenteric bed. However, contact of the polypeptide with the outer surface of the isolated ear artery induced a depression of responses to nerve stimulation.

Angiotensin II and 5-HT at subthreshold concentrations induced a non-specific increase in sensitivity of the rabbit isolated aorta. At contractor concentrations, angiotensin II induced a non-specific depression of responses to vascular agonists. The polypeptide did not inhibit neuronal uptake of noradrenaline. The potentiating action of 5-HT on noradrenaline responses, but not that of angiotensin II, was calcium-dependent. This action of angiotensin II appeared to be a membrane effect, and the involvement of membrane pumps was examined.

The actions of angiotensin II on the rabbit isolated aorta, both direct contractor and indirect potentiation, appeared to be related to the ratio of intracellular to extracellular sodium ions. An attempt was made to provide a comprehensive theory for the mechanism of the potentiating and contractor

actions of angiotensin II in terms of ion fluxes and cellular metabolism in the vascular smooth muscle cell.

Finally, the action of the diuretic drug frusemide was examined on the blood pressure responses of the pithed rat to vascular spasmogens. Neither pretreatment nor acute intra-peritoneal injection had a significant effect on the responses of the preparation. However, acute intravenous injection of frusemide had a specific anti-angiotensin II action in just over half of the animals studied. This action is discussed in terms of the possible ionic fluxes induced by frusemide.

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C O N T E N T S

INTRODUCTION

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SECTION A. The renin-angiotensin system

Tigerstedt & Bergman (1898), found that saline extracts of fresh rabbit kidneys produced a rise in blood pressure when injected intravenously into other rabbits; they named the substance responsible for this effect, renin. These results were confirmed by Bingel & Strauss (1909). However, many other workers could not repeat these results and it was not until the demonstration by Goldblatt, Lynch, Hanzal & Summerville (1934) that hypertension could be produced in the dog by constriction of the main renal arteries that further interest in the role of renin in the physiological control of blood pressure occurred.

The fact that renin acts as an enzyme was discovered by chance. In 1938, Fasciolo, Houssay & Taquini had shown that renal venous blood from hypertensive dogs caused a greater vaso-constriction than did that from normal dogs. Page (1939a) found that renin had no effect in the saline-perfused rabbit ear, causing a vaso-constriction only on perfusion of the ear with plasma. He termed the compound formed on incubation of renin with plasma, angiotonin. Meanwhile, Braun-Menendez, Fasciolo, Leloir & Munoz (1940), found that the pressor compound in the blood of hypertensive dogs was different from renin; they termed the compound hypertensin. When it was realised that these two compounds were, in fact, identical, the two groups of workers agreed on a joint name: angiotensin (Braun-Menendez & Page 1958).

The current state of knowledge of the renin angiotensin system is summarised in fig. 1, and the different components will be described in individual sections.

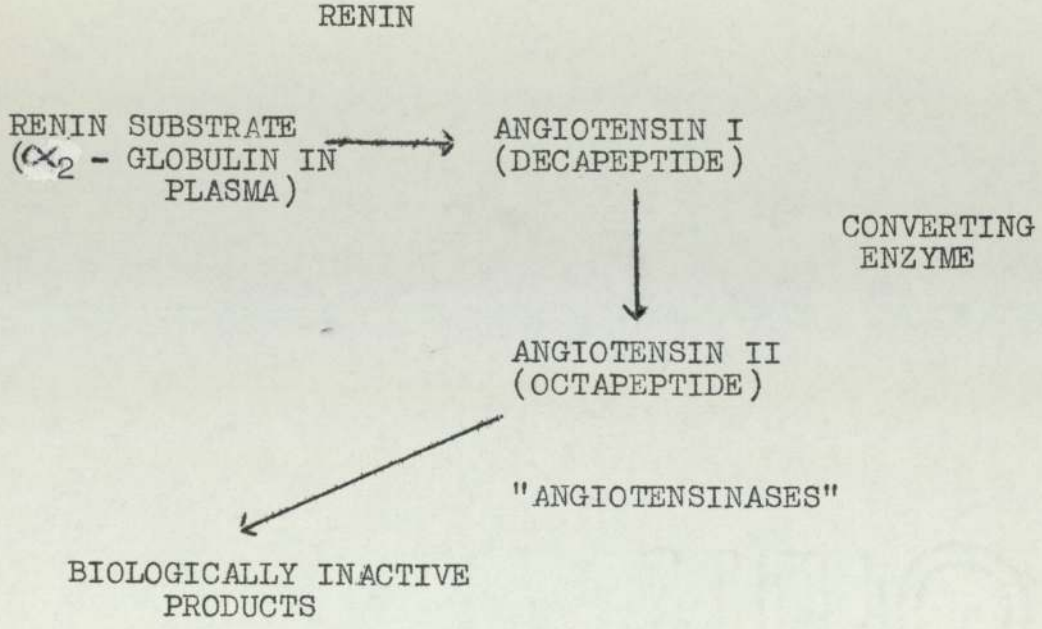

Fig. 1 The renin-angiotensin system

Renin

Hog renin has been found to have a molecular weight of 42,000 to 49,000 (Kemp & Rubin, 1964; Peart, 1966;) and human renin a molecular weight of 42,300 (Lubash & Peart, 1966; Warren & Dolinsky, 1966). Skeggs, Lentz, Kahn & Hochstrasser (1967), suggest that renin isolated from the kidney may not, in fact, be a single compound, but consist of several components separable by chromatography.

Renin is present in the kidneys of all mammals investigated, as well as in birds, amphibia and fish (Nolly & Fasciolo, 1972). The highest concentrations of renin are found in the outer zone of the renal cortex, much less being found in the juxta-medullary area of the cortex, no renin has been observed in the renal medulla (Brown, Davis, Lever, Parker & Robertson, 1966). The juxtaglomerular apparatus is the site of formation and storage of renin in the kidney (Gross, 1971).

Renin also occurs in extrarenal tissues such as the pregnant and non-pregnant uterus of the rabbit, even after nephrectomy (Gross, Schaechtelin, Ziegler & Berger, 1964), the submaxillary gland of mice (Werle, Vogel & Goldel, 1957), and arteries (Dengler, 1956; Gould & Skeggs, 1963; Gould, Skeggs & Kahn, 1964; Genest, Simard, Rosenthal & Boucher, 1969). The latter case may be of especial importance in the control of blood pressure, as an arterial store of renin may be able to produce a local concentration of angiotensin II without build-up of significant levels of angiotensin II in the plasma, as suggested by Khairallah, (1971).

Renin substrate

This is a glycoprotein of the α_2-globulin fraction of plasma. It is thought to originate in the liver as first suggested by Page, (1941). The Substrate disappears from the plasma following hepatectomy or chloroform poisoning (Braun-Menendez, Fasciolo, Leloir, Munoz & Taquini, 1946). The plasma substrate level is also sharply reduced following bilateral adrenalectomy (Helmer & Griffith, 1951), the level being restored to normal by oestrogens, DOCA, cortisone and 11-dehydro-corticosterone (Helmer & Griffith, 1951, 1952).

Ng (1968) found that the formation of angiotensin I from renin substrate occurred in circulating blood and was a rapid process. When dog renin was injected into the venous circulation of a dog, the pressor response began within 15 to 20 sec. (i.e. within one circulation time - Spector, 1956); this would also include conversion of angiotensin I to angiotensin II, as the decapeptide has little intrinsic pressor activity in the whole animal (Page & McCubbin, 1968). Various artificial renin-substrates have been developed by treating plasma proteins with proteolytic enzymes, and it has been found that a synthetic tetradecapeptide substrate reacts with hog or human renin in a similar way to natural plasma substrate (Skeggs, 1960; Montague, Riniker & Gross, 1966). Heparin was found to interfere with the renin-substrate reaction (Sealey, Gerten, Ledingham & Laragh, 1967); it appears to act via a competition with renin-substrate for the enzyme.

Converting enzyme

Skeggs, Kahn & Shumway (1956), first suggested that the decapeptide angiotensin I was converted to the octapeptide angiotensin II by a specific enzyme, which was chloride-dependent. Ng & Vane (1967, 1968) have shown, using the blood-bathed organ technique, that most of the conversion of angiotensin I to angiotensin II occurs in the pulmonary circulation, the blood itself containing little converting enzyme activity.

Recently, Yang, Erdos & Levin (1971), have reported the presence of angiotensin I converting enzyme activity in guinea pig plasma, lung and kidney. They observed that the enzyme has a dual activity; it converts angiotensin I to angiotensin II, and it inactivates bradykinin. Bakhle (1968) partially purified an enzyme from the lungs that converted angiotensin I to II; it was partially inhibited by EDTA, and required chloride ions for its activity. Piqhilloud, Reinharz & Roth (1970) found that human converting enzyme was also inhibited by EDTA.

Angiotensinases

Acute injection of angiotensin II into an experimental animal gives a short lasting (2 to 3 min.) rise in blood pressure. There are various methods of removal of drugs from the circulation, such as inactivation by plasma and tissue enzymes, and tissue sequestration. Angiotensin II is not degraded by a specific angiotensinase, it is broken down by polypeptidases as well as by endopeptidases. The degradation of angiotensin II was summarised by Gross (1963) as can be seen in fig. 2.

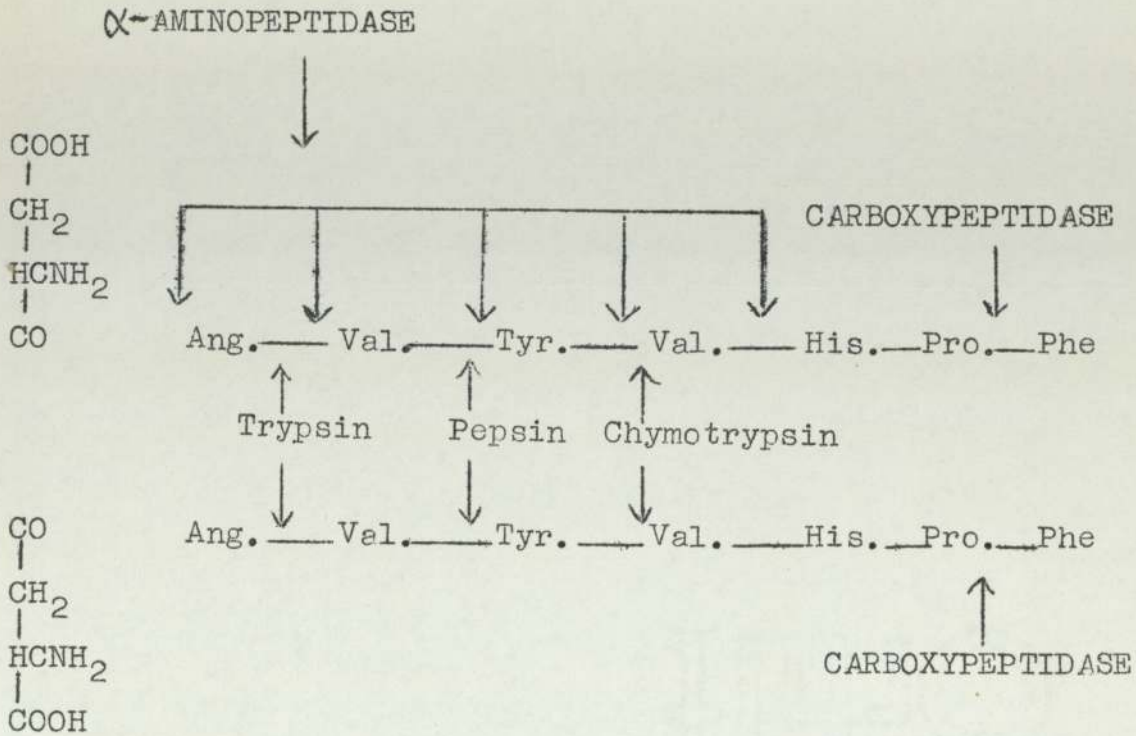

Fig. 2 Degradation of α - and β - angiotensin II amide by "angiotensinases" (after Gross, 1963)

There is no evidence for a carboxypeptidase in plasma, although the enzyme has been isolated from rabbit liver (Johnson & Ryan, 1968) and from kidney and urine (Yang, Erdos & Chiang, 1968). The latter authors have termed this enzyme angiotensinase C.

Khairallah, Bumpus & Smeby (1963), found a plasma amino-peptidase requiring Ca^{++} ions, which was inhibited by EDTA or sodium pyrophosphate and Itskovitz & Miller (1967) reported an inhibiting action of δ-amino caproic acid. This enzyme was termed angiotensinase A. Regoli, Riniker & Brunner (1963), described a plasma aminopeptidase which split the angiotensin II molecule into two tetrapeptides. This enzyme was inhibited by DFP (Pickens, Bumpus, Lloyd, Smeby & Page, 1965) and was termed angiotensinase B.

Ferreira & Vane (1967), and Ng (1968), found that on passage of angiotensin II through various organs of the dog, over half of the angiotensin II was removed from the blood during one circulation ; passage through the lungs, however, resulted in no loss of angiotensin II. However, Hodge, Ng & Vane (1967), and Ng (1968) found that the biological half life of the peptide in blood in vitro at 37C ranged between 140 and 280 sec. As one circulation time in the dog takes only 15 secs. (Spector, 1956), it is obvious that the vascular tissue is playing an active part in either removal or breakdown, or both, of angiotensin II.

Haas, Goldblatt, Lewis & Gipson (1968), reported the isolation of an angiotensin II - cofactor from normal serum of man, cow and dog. They found that this cofactor prevented the loss of angiotensin II from the circulation on passage through the femoral arterial bed and hind quarters of a dog. They concluded that angiotensin II is being taken up onto tissue receptors, and that the cofactor prevents the binding of angiotensin II with these "silent" receptors.

Tissue homogenates, such as those of lung (Bakhle, 1968) adrenal gland (Bumpus, Smeby, Page & Khairallah, 1964) and liver, kidney, skeletal muscle and intestines all inactivated angiotensin II (Itskovitz & Miller, 1967), probably due to the fact that tissues are rich sources of proteolytic enzymes. However, although lung homogenates destroy angiotensin II, it is known that in vivo angiotensin II is not destroyed in the pulmonary circulation (Ng & Vane, 1967, 1968).

The Pharmacology of angiotensin II

a) Effects on non-vascular smooth muscle

Intestine

Isolated intestinal preparations taken from most species contract in response to angiotensin II, but are not uniform in either the qualitative or quantitative nature of the response. The guinea pig ileum responds to angiotensin II in the range 10^{-9} to 10^{-8} g/ml.,

with a slight (30 to 40 sec.) delay between contact of the tissue with the drug, and the commencement of the response. (Gross & Turrian, 1960; Khairallah & Page, 1961, 1962; Bisset & Lewis, 1962). The rabbit ileum also contracts in response to angiotensin II, (Robertson & Rubin, 1962), as do the ilea of the rat, mouse and Mongolian gerbil (Goldenberg, 1967), although none of these ilea were as sensitive as that of the guinea pig. The rat isolated colon also responds to angiotensin II (Bisset & Lewis, 1962), and this organ has been used in the bioassay of angiotensin II (Regoli & Vane, 1964). Ng (1968) found that the rat stomach strip and the rabbit rectum responded only weakly to the peptide, and the chicken rectum and rat jejunum did not respond at all. However, recently, Rioux, Park & Regoli (1973) have induced dose-response curves to angiotensin II on the rat stomach strip in the range 10^{-9} to 10^{-5}g/ml. Angiotensin II also inhibits ciliary movements of the frog oesophagus, an atropine-like action (Kiran & Khairallah, 1968).

Uterus

Uteri from rats either in oestrus or pretreated with stilboestrol reacted to angiotensin II with a dose dependent contraction, (Gross & Turain, 1960; Khairallah & Page, 1963; Paiva & Paiva, 1960). Responses to angiotensin I were minimal, (Bumpus, Smeby & Page, 1961) indicating that there is little converting enzyme activity in this tissue.

Other smooth muscle

Guinea pig seminal vesicle and the dorsal muscle of the leech appear to be insensitive to the contractile action of angiotensin II (Gross & Turain, 1960). Guinea pig vas deferens also shows no sensitivity to the direct contractile activity of angiotensin II, however there is a potentiation of the responses to hypogastric nerve stimulation in the presence of angiotensin II (Benelli, della Bella & Gandini, 1964). Turker & Kiran (1964) found that concentrations of angiotensin II up to 10^{-4} g/ml had no effect on tracheal muscle from cats, dogs, rats, rabbits and guinea pigs. In isolated lungs of the guinea pig angiotensin II increased the resistance to inflation, but was less active in this respect than kallidin (Bhoola, Collier, Schachter & Shorley, 1962). Angiotensin II in concentrations above 10^{-8} g/ml increased the tone of rat isolated renal pelvis muscle, a consequence of which, in vivo, would be a reversible obstruction of urine outflow from the kidney. (Finberg & Peart, 1970).

b) Effects of angiotensin II on vascular smooth muscle

Isolated arteries

Spirally-cut thoracic aortae from the rabbit, first analysed pharmacologically by Furchgott & Bhadrakom (1953), have been used extensively to assay angiotensin II (Helmer, 1964). The reported time of response varies; Bohr & Uchida (1967) and Khairallah, Page, Bumpus & Turker (1966a) found a response beginning within 30 sec. of contact of angiotensin II with the tissue, and ending after 1 to 2 min. However, Helmer (1957, 1964) reported a slow onset of contraction (2 to 4 min.) and a maximum response reached after 10 to 20 min.

The major site of action of angiotensin II in the vascular bed is on peripheral resistance vessels (Mellander & Johansson, 1968). Spirally-cut isolated arteries from various species show a varying sensitivity to the constrictor effect of angiotensin II. Khairallah et al (1966a) found that the cat carotid artery responded to 10^{-9} g/ml of angiotensin II, whereas dog carotid and rat aorta both required concentrations above 10^{-7} g/ml; responses of rabbit aorta and sheep carotid were intermediate in sensitivity. Ng & Vane (1967, 1968) reported a constrictor action of angiotensin I on rabbit aorta, though it seems likely that this is due to a conversion to the octapeptide, rather than a direct action, as Bumpus, Smeby & Page (1961) have reported the presence of converting enzyme activity in the rat aorta.

Bohr & Uchida (1967) found that spirally-cut canine resistance vessels - coronary, mesenteric and skeletal vessels all responded to angiotensin II, the coronary vessels exhibiting rapid tachyphylaxis. However, renal resistance vessels did not respond to angiotensin II, even though they did to KCl and adrenaline.

Isolated veins

Contractions of canine venous strips were recorded with concentrations of 5×10^{-9} g/ml. of angiotensin II in hepatic portal mesenteric and lobar pulmonary veins, whereas femoral or axillary veins and vena cava sections were not contracted by concentrations 100x greater than this (Somlyo & Somlyo, 1964). Compared with noradrenaline and histamine, angiotensin II has much less activity on

isolated venous strips, and exhibits pronounced tachyphylaxis (Somlyo & Somlyo, 1966). The rat isolated portal vein was found to be the vein most sensitive to the direct constrictor action of angiotensin II, spontaneous rhythmic contractions were enhanced by concentrations of 10^{-11} g/ml of angiotensin II (Bohr & Uchida, 1967). The response of the rat portal vein to angiotensin II has recently been fully characterised by Blair-West & McKenzie (1971)

Vascular beds (in vivo and in vitro)

Many investigators have observed that the potency of angiotensin II varies within different vascular beds. Barer (1961) found that angiotensin II increased the resistance in every vascular bed into which it was injected directly. He observed a strong vasoconstrictor action on the renal and mesenteric beds, whereas angiotensin II had only a weak effect on coronary and pulmonary beds.

Using both the dog perfused forelimb and the perfused mesenteric vascular bed, Haddy, Molnar, Borden & Texter (1962) found that angiotensin II on a weight for weight or molecular basis was a stronger vasoconstrictor agent than either noradrenaline or 5-HT. They found that forelimb vascular volume decreased less during intra-arterial administration of angiotensin II than during intra-arterial administration of noradrenaline; this difference indicated a lesser ability of angiotensin II to contract veins. Bohr & Uchida (1967) examined

resistance vessels from the mesentery and brain of the rabbit. They found that these vessels were alike in the concentrations required for threshold constrictor response to KCl, angiotensin II and plasma, but cerebral vessels had a higher threshold for noradrenaline, adrenaline and 5-HT, and a lower threshold for the response to vasopressin.

Abboud (1968) examined the responses of various vascular beds in the anaesthetised dog to angiotensin II, noradrenaline and 5-HT. The resistance vessels were relatively more sensitive to angiotensin II than noradrenaline, whereas capacitance vessels were more sensitive to noradrenaline than angiotensin II. He observed that the constrictor effect of angiotensin II appeared to be related to the degree of sympathetic tone, but unlike 5-HT the constrictor action of angiotensin II was augmented at high sympathetic tone. Kormano (1970) observed a vasoconstriction of rat testicular blood flow in response to 5-HT, and a total occlusion at higher concentrations; angiotensin II caused only vasoconstriction, and not total occlusion of the circulation.

Angiotensin II applied topically to veins in the dog, had no effect even in high concentrations, whereas noradrenaline caused marked vasoconstriction (Rose, Kot, Cohn, Freis & Eckert, 1962). The relatively low activity of angiotensin II on veins is also shown by the absence of a marked venoconstrictor effect on the conjunctival veins after instillation into the eye of the rabbit (Gross & Bock, 1962).

Blood pressure of whole animal

The response to a single intravenous injection of angiotensin II is the same in all species of animals studied - after a lag period of 20 to 30 sec., blood pressure rises sharply, reaches a maximum within 1 to 2 min., then diminishes to the original level within 3 to 5 min. (Page, McCubbin, Schwarz & Bumpus, 1957; Bock & Gross, 1961). The response to angiotensin II is repeatable, though large concentrations induce tachyphylaxis (Bock & Gross, 1961). Angiotensin II amide (Hypertensin-Ciba) is about 10 to 20 times as active (on a weight/weight basis) a constrictor agent as noradrenaline, and pulse pressure may increase since the diastolic pressure is raised less than the systolic (Bock, Krecke & Kuhn, 1958; Bock & Gross, 1961).

Dickinson & Lawrence (1963) and Dickinson & Yu (1967) reported a slow, progressive elevation of blood pressure during intravenous infusions of subpressor concentrations of angiotensin II into the conscious dog. On cessation of infusion, the blood pressure reverted rapidly to pre-infusion values. Very high concentrations of angiotensin II (1.5×10^{-5} g/kg/min.) produced a sharp increase in blood pressure which fell after a few minutes to control values despite continued infusion of the peptide (Bock & Gross, 1961).

Unlike the dog, the rat responds with a lasting rise in blood pressure to infusions over 8 to 12 days and acute hypertensive vascular disease indistinguishable from acute experimental hypertension has been observed (Koletsky, Rivera-Velez & Pritchard, 1965).

c) Sympathetic interactions

There have been many reports of interactions between angiotensin II and the sympathetic nervous system, and to a lesser extent, between angiotensin II and exogenous noradrenaline. Recent reviews by Lowe & Scroop (1970), Papanicolaou, Meyer & Milliez (1970), and Starke (1972a) indicate that the site of interaction may be at several levels, both central and peripheral. The central actions of angiotensin II will be dealt with under a different section, as will actions on ganglia.

Direct release of endogenous noradrenaline

Liebau, Distler & Wolff (1965) and Distler, Liebau & Wolff (1965) observed a reversal of angiotensin II - induced tachyphylaxis in mammalian isolated aortae (cow, pig, rat and man) after incubation of the aortae with noradrenaline. This reversal of tachyphylaxis was prevented by cocaine, and hastened by pretreatment of the animal with reserpine; there was a cross-tachyphylaxis between tyramine and angiotensin II. Suzuki & Matsumoto (1966) observed a reduction (30%) of the constrictor response of rabbit isolated aortae

to angiotensin II in the presence of either α-blocking drugs or reserpine, and a potentiation in the presence of MAO and COMT inhibitors. Schumann & Guther (1967) using guinea pig aorta, found a 70% inhibition of responses to angiotensin II by cocaine and phentolamine and a cross-tachyphylaxis between tyramine and angiotensin II; a similar reduction of the response to angiotensin II by phentolamine was seen in rat aorta. They also report a reduction in size of the response to angiotensin II in guinea pig aortae taken from reserpine - pretreated animals, as the response is greater after incubation of the tissue with noradrenaline. However, a comparison of the responses to angiotensin II in both types of aortae yields no significant difference, i.e. the authors are confusing a potentiation of responses to angiotensin II by noradrenaline with what they interpret as an initially reduced response in treated tissues.

Turker & Karahuseyinoglu (1968) found that concentrations of cocaine (10^{-5} to 1.5×10^{-4} g/ml) which totally blocked the response to tyramine on the rabbit aorta had no effect on the responses to angiotensin II. They suggested that, at the concentrations used by the previous authors, (greater than 10^{-3} g/ml) cocaine was having a non-specific action. Khairallah et al (1966a) using the rabbit aorta, failed to induce a reversal of angiotensin II tachyphylaxis on incubation of the tissue with noradrenaline, as did Palaic & Lemorvan (1971) using the guinea pig aorta. Sakurai & Hashimoto (1965) found that vascular preparations taken from reserpinized rabbits were sensitive to angiotensin II, and in some cases, exhibited a greater sensitivity than controls.

Gascon & Vaillancourt (1969) found that the isolated guinea pig seminal vesicle responds to angiotensin II only after incubation of the tissue with adrenaline. There was, however, no cross tachyphylaxis between angiotensin II and tyramine. They suggest that in this tissue angiotensin II acts by releasing catecholamines from extra-neuronal storage sites. On the renal vasculature of the rabbit, McGiff & Fasy (1965) found that the constrictor action of angiotensin II was blocked by reserpine, guanethidine and bretylium, suggesting that in this tissue angiotensin II was acting by release of neuronal catecholamines.

Facilitation of endogenous noradrenaline release

Benelli, della Bella & Gandini (1964) found that although angiotensin II induced no response of the guinea pig vas deferens itself, it did potentiate the response to hypogastric nerve stimulation. It did not affect the responses to exogenous noradrenaline and actylcholine. However, Thoenen, Hurlimann & Haefely (1965) observed a potentiation of responses to postganglionic stimulation of the cat spleen in the presence of angiotensin II, but could not detect facilitation of noradrenaline release. They proposed a post-synaptic site of interaction between angiotensin II and the sympathetic nervous system. In the isolated central ear artery of the rabbit, responses to angiotensin II were potentiated during release of endogenous amines by either nerve stimulation or tyramine, the potentiation being abolished by reserpine, guanethidine and phentolamine (Day & Owen, 1968). Conversely, responses to nerve

stimulation and tyramine were depressed in the presence of angiotensin II. However, in the pithed rat, Day & Owen (1969) found that responses to both nerve stimulation and tyramine, but not those to noradrenaline, were potentiated during an infusion of angiotensin II.

Hughes & Roth (1969, 1971) measured the outflow of noradrenaline from the rabbit portal vein during nerve stimulation, and found that, in the presence of angiotensin II, this outflow was significantly increased. Bell (1972), using the guinea pig isolated vas deferens and uterine artery observed a greater potentiation of low frequency than high frequency nerve stimulation in the presence of angiotensin II.

Effect on catecholamine biosynthesis

Panagiotis & Hungerford (1966) first observed a possible influence of angiotensin II on the metabolism of noradrenaline. They found that angiotensin II caused a marked increase in osmophilic granules in the vesicles of sympathetic nerve endings in the pineal gland. As these granules are associated with noradrenaline, they suggest that the pressor activity of angiotensin II may be influenced by an increase in adrenergic material in the endings of the sympathetic nerves.

Boadle, Hughes & Roth (1969), using guinea pig atria and vas deferens, found a significant increase in amine content of the tissues after incubation with angiotensin II at concentrations as low as 10^{-9} g/ml; Roth & Hughes (1972) correlated an acceleration of protein synthesis

by angiotensin II with it's effect on catecholamine biosynthesis. They found an inhibition of the action of angiotensin II by protein synthesis inhibitors, oligomycin and actinomycin D.

Effect on uptake of noradrenaline

Palaic & Khairallah (1967a) reported that infusion of angiotensin II in concentrations ranging from $8x10^{-9}$ g/ml to $8x10^{-7}$ g/ml over a period of one hour significantly inhibited the uptake of noradrenaline into rat brain. In a further report, Palaic & Khairallah (1967b), angiotensin II at a concentration of $2x10^{-5}$ g/ml was shown to inhibit noradrenaline uptake into slices of rat brain, spleen and aorta. Peach, Bumpus & Khairallah (1969) reported a 50% to 80% inhibition of noradrenaline uptake into the rabbit isolated heart during infusions of angiotensin II in concentrations ranging from $5x10^{-11}$ g/ml to $2x10^{-9}$ g/ml. Palaic & Panisset (1969) found a potentiation of the responses to exogenous noradrenaline in the perfused cat mesenteric bed in the presence of angiotensin II; however, they could record no significant inhibition of uptake of noradrenaline until 10x this concentration of angiotensin II was used; suggesting that an inhibition of neuronal uptake of noradrenaline was only part of the mechanism by which angiotensin II was acting. Khairallah, Papanicoloau, Davila, Glende & Meyer (1971) observed an inhibitory action of angiotensin II on the uptake of noradrenaline into the heart and several other organs on injection into the pithed rat. The adrenal medulla was an exception - catecholamine uptake was increased, rather than decreased, in the presence of angiotensin II. Davila & Khairallah

(1970) found that both ouabain and angiotensin II decreased the uptake of noradrenaline and metaraminol into rat isolated auricles, the action of cuabain, but not that of angiotensin II, being calcium dependent.

Other workers have failed to observe an inhibition of tissue noradrenaline uptake in the presence of angiotensin II. Pals, Fulton & Masucci (1968) reported that the uptake of noradrenaline into the heart of the pithed rat was reduced by cocaine and desipramine, but was unaffected during an infusion of angiotensin II ($3x10^{-7}$ g/kg/min.). Similarly, Schumann, Starke, Werner & Hellerforth (1970) found that noradrenaline uptake into the rabbit isolated perfused heart was not affected by concentrations of angiotensin II up to 10^{-7} g/ml. Angiotensin II (10^{-6} g/ml), unlike cocaine, failed to prevent the reversal of tyramine tachyphylaxis by noradrenaline in the rat isolated vas deferens (Trager, Kreye & Gross, 1972).

Adrenal medulla

Angiotensin II was first shown to release catecholamines from the adrenal medulla by Braun-Menendez, Fasciolo, Leloir & Munoz (1940). Feldberg & Lewis (1964) investigated the action of angiotensin II on the feline adrenal medulla. They found that acute injection of 10^{-6} g/ml of angiotensin II into the central stump of the celiac artery raised blood pressure and contracted the denervated nictitating membrane in the presence of intact adrenals.

Staszewska-Barczak & Konopka-Rogatko (1967) confirmed the findings of Feldberg & Lewis (1964) and found that the adrenaline-releasing action of angiotensin II is not blocked, but potentiated, by the ganglion-blocking agent pentolinium. They suggest that angiotensin II acts directly on the adrenal-medullary cells and not via a stimulation of nerve endings in the gland. In perfused cat adrenal glands, Poisner & Douglas (1966) found that calcium ions were necessary for release of catecholamines by angiotensin II. Robinson (1967) confirmed this in the dog and Douglas, Kanno & Sampson (1967), using intracellular micro-electrodes, reported that angiotensin II depolarised the chromaffin cells. Morphine, hexamethonium, 5-HT and histamine did not modify the effect of angiotensin II (Lewis & Reit, 1966), suggesting that angiotensin II acts as a non-nicotinic ganglion stimulant, directly on the adrenal chromaffin cells.

d) Interaction of angiotensin II with the parasympathetic nervous system and autonomic ganglia

Robertson & Rubin (1962) reported a spasmogenic action of angiotensin II on the isolated intestine of the rabbit and guinea pig. This contraction was enhanced by anticholinesterases and reduced by atropine and by botulinum toxin. Their results suggest that part of the angiotensin II induced contraction is mediated via the cholinergic nerve-endings in the tissue. Khairallah & Page (1963) reported the presence of acetylcholine in the muscle chamber during contraction of isolated intestinal strips induced by

angiotensin II. Goldenberg (1967) reported a cholinergic component in the response to angiotensin II of mouse and gerbil ilea, but the rat ileal response was not inhibited by atropine or morphine. Regoli & Vane (1964) found that the angiotensin II-induced contraction of the rat colon was not inhibited by morphine, i.e. it was not neuronally mediated. Beleslin (1968), using guinea pig ileum, concluded that angiotensin II had three sites of action; ganglionic cells, postganglionic nerve endings and intestinal smooth muscle cells. There is an isolated report of angiotensin II having an atropine like action; Kiran & Khairallah (1968) found that the ciliary motility of frog oesophagus was initiated by acetylcholine, reduced by atropine and increased by physostigmine; angiotensin II inhibited the ciliary motion.

Feldberg & Lewis (1964), first reported that angiotensin II, administered by close intra-arterial injection to the superior cervical ganglion of the anaesthetised cat, caused a contraction of the ipsilateral nictitating membrane. The response was abolished by ganglionectomy or by section of the postganglionic, but not the preganglionic, trunk. Lewis & Reit (1966) found that this ganglion-stimulating action of angiotensin II was abolished by morphine and that during the depolarising phase of nicotine-induced ganglion blockade, the response to angiotensin II was blocked; however, during the competitive phase of nicotinic blockade, the stimulating action of angiotensin II was enhanced, i.e. angiotensin II acts as a non-nicotinic ganglion stimulant.

Haefely, Hurlimann & Thoenen (1965) and Panisset, Biron & Beaulnes (1966) report paradoxical actions of angiotensin II on the ganglion. Low concentrations of angiotensin II, less than 10^{-11} g., inhibited contraction of the nictitating membrane in response to preganglionic electrical stimulation, whereas larger concentrations facilitated ganglionic transmission. Panisset (1967) found an increased release of acetylcholine from perfused ganglia in response to nerve stimulation in the presence of angiotensin II. Angiotensin II also caused a release of acetylcholine from parasympathetic ganglia in submaxillary glands. Other ganglion stimulating actions of angiotensin II are described in the next section (HEART).

e) Effects of angiotensin II on myocardial muscle

Many preparations have been utilised to elucidate the mechanism of action of angiotensin II on the heart; these include in vitro preparations of heart muscle (Koch-Weser, 1964), the Langendorff heart preparation (Peach et al, 1969; Schumann et al, 1970), heart-lung preparations (Fowler & Holmes, 1964) and modified whole animal preparations (Farr & Grupp, 1967, 1971).

Koch-Weser (1964) used two feline isolated heart muscles; the kitten papillary muscle from the ventricle, and atrial muscle. He found a positive inotropic effect of angiotensin II on ventricular myocardium in the dose range 10^{-10} to 10^{-5} g/ml. Unlike sympathomimetic amines, angiotensin II produced no decrease in the duration of the active state, therefore,

ventricular systole is not shortened by angiotensin II. Low concentrations of angiotensin II had a greater inotropic action than equimolar concentrations of noradrenaline, but the maximum effect available with noradrenaline was almost twice that with angiotensin II. He found little action of angiotensin II on cat atrial muscle, or on electrical activity of the S-A node. James (1965) found no chronotropic effect on infusion of angiotensin II directly into the sinus node artery of the dog.

Koch-Weser (1965) investigated the possible sympathetic interaction of angiotensin II in the isolated kitten papillary muscle. He found that reserpine pre-treatment almost abolished the positive inotropic action of tyramine, but slightly potentiated that of angiotensin II; β-receptor blockade (nethalide) blocked tyramine, but had no effect on angiotensin II. His results indicate that the positive inotropic action of angiotensin II on feline isolated ventricular muscle does not result from release of cardiac catecholamines. These results are in agreement with those of Lefer (1970) and Fowler & Holmes (1964), who found that isolated papillary muscles from reserpine-pretreated kittens were as responsive to angiotensin II as those from non-reserpinized animals.

Beaulnes (1964) felt that the positive inotropic response to angiotensin II was due to a liberation of catecholamines from the heart, as β-blocking agents inhibited the inotropic response. Drimal (1969)

used the isolated papillary muscle of the young dog, and with an angiotensin II dose range of 10^{-8} to 10^{-5} g/ml found an increase in force, velocity and resting tension, while the duration of the active state of the heart muscle remained unchanged. From the results obtained using adrenergic receptor blocking drugs, he concluded that the inotropic action of angiotensin II was mediated through the release of catecholamines from the tyramine-sensitive structures in the myocardium.

Compared with the effect on papillary muscle or atria, the inotropic effect on the isolated perfused heart is less marked, or absent. In the guinea pig heart, there was a reduction in amplitude in response to angiotensin II (Heeg & Meng, 1965), while in the isolated rabbit or cat heart, a slight increase in amplitude was seen (Meier, Tripod & Studer, 1958). Smyth (1961), using the rabbit isolated heart, also reported mild inotropic effects (3% to 5% increase) with concentrations of 10^{-6} and $5x10^{-6}$ g/ml of angiotensin II; larger concentrations of angiotensin II - 10^{-3} and $2x10^{-3}$ g/ml, were depressant. He also reported that angiotensin II infusions did not potentiate the inotropic effect of adrenaline, an interesting observation in the light of the recent report by Inoue, Smulyan, Mucha & Eich (1973) of a potentiating action of angiotensin II on the inotropic effect of glucagon in the rabbit isolated heart. Bianchi, De Schaepdryver, de Vleeschhouwer & Preziosi (1960) reported a biphasic action; an

initial reduction and subsequent increase in the contractile force of the rabbit and guinea pig heart in the presence of high concentrations of angiotensin II. Dempsey, McCallum, Kent & Cooper (1971), using the isolated cat heart, demonstrated a direct positive inotropic action of angiotensin II, in the concentration range 10^{-10} to 10^{-6} g/ml.

Fowler & Holmes (1964), using the dog heart-lung preparation, found a positive inotropic effect of angiotensin II; prior reserpinisation of the animal had no effect on this. They also found increased coronary vascular resistance after angiotensin II. In the anaesthetised dog with baroreceptor reflexes blocked, angiotensin II had a positive inotropic and chronotropic action (Krasney, Paudler, Smith, Davis & Youmans, 1965) neither actions were blocked by blockade or removal of cardiac ganglia, but the chronotropic action was blocked by either bretylium or β-blocking drugs. They concluded that the inotropic action of angiotensin II is largely direct, whereas the chronotropic action is largely, or entirely, indirect.

Farr & Grupp (1967) observed positive inotropic and chronotropic responses to angiotensin II (10^{-6} g/kg) in the anaesthetised ganglion-blocked dog, which excluded the possibility of an action mediated via the CNS. Angiotensin II given rapidly intravenous to the anaesthetised dog in the concentration range 10^{-7} to 10^{-6} g/kg increased the heart rate and

contractile force. Both effects were reduced by β-blockade, reserpinisation or de-centralisation, but not by ganglion blockade or bilateral adrenalectomy (Farr & Grupp, 1971). Even though angiotensin II induced a marked increase in plasma and myocardial amine levels in animals with intact adrenals (Peach & Ford, 1968) which would be expected to contribute to the tachycardia.

Farr et al (1968) demonstrated that the major site of ganglionic synapse of sympathetic fibres affecting heart rate and right ventricular contractile force in the dog are situated in the caudal cervical ganglion, and not in the stellate as in the cat. Farr & Grupp (1971) found that the inotropic and chronotropic responses to angiotensin II were significantly reduced by crushing the right cervical caudal ganglion, or by cutting its postganglionic sympathetic fibres; this did not occur with any other ganglion.

In conscious man (Bianchi, de Schaepdryver, de Vleeschhouver & Preziosi, 1960; Page & Olmsted, 1961; Olmsted & Page, 1965; Page & McCubbin, 1965) and animals (Westfall & Peach, 1965) angiotensin II induced a reflex bradycardia mediated by the vagus, varying directly with the concentration of angiotensin II. The bradycardia was abolished by atropine and anaesthesia, so that angiotensin II administered to anaesthetised animals leads to the tachycardia discussed above. (Nishith, Ganguly, Ramanathan & Sreepathi Rao, 1966).

f) Effect of angiotensin II on the central nervous system

Bickerton & Buckley (1961) first demonstrated conclusively a centrally-mediated increase in blood pressure in response to angiotensin II. They infused angiotensin II into the cerebral circulation of a dog whose head was connected to it's body by nerves alone; the resultant pressor response was blocked by the adrenergic blocking drug piperoxan. Severs & Daniels-Severs (1973) have recently written a review on the central actions of angiotensin II. In the conscious cat, Day & Owen (1970) found results suggesting that the pressor response resulting from acute intravenous injection of angiotensin II does not involve an action via the central nervous system.

Cooling & Day (1973) have shown that angiotensin II administered intracerebroventricularly into the conscious cat induces drinking; the dipsogenic action of angiotensin II has previously been reviewed by Fitzimmons (1972)

g) Effect of angiotensin II on ions and osmolarity

Many reports have linked angiotensin II with a variation in tissue cation levels.

Calcium

Singh (1964) observed that frog isolated stomach muscle and heart no longer responds to angiotensin II in the absence of calcium ions. The response of the rat uterus to angiotensin II was altered in character in the absence of calcium, being of the nature of a contracture rather than the normal twitch response, while the guinea pig ileum continued to respond unless there was a total absence of calcium, when no response to angiotensin II could be elicited (Khairallah, Vadaparamil & Page, 1965). The calcium dependency of angiotensin II-induced responses of perfused mesenteric arteries (Burks , Whitacre & Long, 1967) perfused adrenal glands (Robinson, 1967), and rabbit perfused isolated kidney (Kline & Buckley, 1969) has also been demonstrated.

In the presence of high concentrations of angiotensin II, an increased uptake of calcium ions across the cell membrane has been reported in guinea pig isolated taenia coli (Shibita, Carrier & Frankenheim, 1968) and a shift of calcium from extra-cellular to intracellular sites in the rat isolated uterus (Khairallah, 1970). Sullivan & Briggs (1968) reported that lowering the calcium concentration to 0.15mM had no effect on the response of the rabbit isolated

aortic strip to angiotensin II, but the addition of 0.5mM manganese greatly reduced the response. They suggested that manganese was inhibiting the calcium permeability. Angles d'Auriac, Baudouin & Meyer (1972) found in rabbit isolated aorta that angiotensin II in concentrations ranging from 10^{-9} to 10^{-7} g/ml caused an inhibition of calcium binding to the cell membrane at the lower concentrations, whilst at the higher concentrations, it increased release of calcium from the cell membrane. Limas & Cohn (1973) suggested that angiotensin II acts on the Ca^{+}/ Na^{+} pump in the canine aorta to induce a contraction via stimulation of the pump, and hence increase intracellular calcium.

In the whole animal, Daniels, Severs & Buckley (1967) found that infusion of angiotensin II either prevented skeletal uptake or caused a shift of the tracer dose of radioactive Ca^{2+} into the soft tissue, in the anaesthetised rat.

Sodium and Potassium

Napodano, Caliva, Lyons, De Simone & Lyons (1962), found that acute reduction of the external sodium ion concentration, causing a decrease in the Na^{+}_{O}/Na^{+}_{I} gradient, enhanced the response of rabbit isolated aorta to angiotensin II. An acute increase in the external sodium ion concentration, with a probable increase in the Na^{+}_{O}/Na^{+}_{I} gradient, lessened the response to angiotensin II. Khairallah et al (1965) found that reduction of external sodium concentration by one half

inhibited response of guinea pig ileum to angiotensin II, but sodium had to be reduced to 10% of control values before the response of the rat isolated uterus to angiotensin II was abolished. The authors conclude that this sensitivity to alterations in sodium concentration reflects the relative neuronal involvement in the response to angiotensin II; in the guinea pig ileum the response is largely neuronally mediated, whereas in the rat uterus it is direct. Blair-West & McKenzie (1966) and Blair-West, Harding & McKenzie (1967) confirmed these findings in guinea pig ileum, and found that increasing the sodium concentration of the bath increased the response to angiotensin II; they concluded that the variation in activity of the tissue with variation in external sodium ion concentration was due to an altered sensitivity of the smooth muscle cells themselves, rather than the parasympathetic ganglia. Lefer (1970) found that decreasing the external sodium ion concentration reduced the positive inotropic effect of angiotensin II on cat isolated papillary muscle, and increasing it enhanced the positive inotropic effect. Harris & Palmer (1972) found that increasing the external sodium concentration of perfused rabbit isolated arterial segments resulted in an increased sodium content of the tissue, and an increased response of the tissues to noradrenaline, histamine electrical stimulation and angiotensin II. However, Blair-West et al (1968) observed an inhibition of responses to angiotensin II in the rabbit isolated ear both by low and high sodium perfusates.

In the whole animal the situation is more complex. Doca saline hypertensive rats are more responsive to vasoconstrictors, including angiotensin II (Gross & Lichtlen, 1958) but this may be due to factors other than alteration of ionic fluxes, e.g. structural factors (Tobian & Redleaf, 1957). Kreiger & Hamilton (1958) examining the cardiovascular activity of acutely nephrectomised dogs receiving peritoneal lavage, found that glucose-insulin infusions lowered serum potassium and reduced responses to noradrenaline, adrrenaline and angiotensin II to one third of control levels. After KCl administration responses returned almost to control levels. Stouder & Wathen (1972) found that nephrectomy or ureteral ligation in the rat induced a significant enhancement of the response to angiotensin II. This enhancement was accompanied by a rise in plasma potassium and a marked fall in the plasma Na/K ratio. However, Strewler, Hinrichs, Guiod & Hollenberg (1972) found that rabbits consuming a low sodium diet exhibited a reduced responsiveness to angiotensin II of their limb vessels perfused in vivo and thoracic aorta in vitro; the response to noradrenaline was potentiated in both preparations. It may be that the enhanced pressor activity to exogenous angiotensin II seen in nephrectomised animals is simply due to low endogenous levels of angiotensin II.

The involvement of ions in vascular reactivity is more thoroughly examined in Sec.B of this INTRODUCTION.

Reports of an action of angiotensin II on sodium and potassium fluxes are variable. Friedman & Friedman (1964) found that constrictor concentrations of angiotensin II, in common with those of other agonists, caused a shift of sodium ions into and potassium ions out of vascular smooth muscle cells. Daniel (1965a)found that large concentrations of angiotensin II ($5x10^{-8}$ to $5x10^{-7}$ g/ml) effectively inhibited reaccumulation of potassium by rabbit aortic strips recovering from sodium loading, but the sodium loss was unaffected. Rorive, Hagemeijer & Schoffeniels (1967) reported a transient increase in potassium efflux from perfused rat aortic preparations in response to angiotensin II, whilst Turker, Page & Khairallah (1967) found that sub-maximal concentrations of angiotensin II, but not 5-HT or bradykinin, increased sodium efflux from isolated canine carotid arteries and rat uterus. Khairallah (1971), however, felt that the efflux was occurring during a period of repolarisation of the membrane, even though Turker et al (1967) observed a blockade of the angiotensin II-induced contraction of the uterus by ouabain. Szalay (1969) reported an inhibition of potassium accumulation in pig isolated adrenals during incubation with a very high concentration of angiotensin II ($2x10^{-4}$ g/ml). In perfused rat caudal artery, Guignard & Friedman (1971) found that sub-threshold concentrations of angiotensin II caused a loss of potassium from the perfusing medium and a gain in sodium, whereas constrictor concentrations caused a smaller gain in sodium and a non-significant change in potassium levels.

In vivo, Villamil, Nachev & Kleeman (1970) observed that longterm (5 to 6 weeks) treatment of dogs with angiotensin II resulted in an increased sodium level in the arterial wall.

Hydrosmotic effect of angiotensin II

Angiotensin II has been found to increase water uptake through the skin of the toad when injected into the whole animal (Coviello, 1970); however, due to the ADH-releasing action of angiotensin II (Bonjour & Malvin, 1970), it was not possible to describe this as a direct action of angiotensin II. Experiments using isolated sacs of toad skin showed that angiotensin II at a concentration of 2×10^{-7} g/ml increased osmotic water flow, (Coviello & Brauckman, 1973). Previously, Coviello (1972) found that angiotensin II in concentrations of 2×10^{-9} to 10^{-7} g/ml produced a significant increase in water permeability in the toad bladder.

Tissues from other species also show responses to angiotensin II. Crocker & Munday (1967) demonstrated that angiotensin II increased fluid transfer in the rat jejunum, and Davies, Munday & Parsons (1970) reported a dose-dependent stimulatory or inhibitory effect on fluid transfer in rat colon. An inhibitory effect was also found in rabbit gall bladder (Frederikson & Leysaac, 1969).

Effects on the kidney

Exogenous angiotensin II affects both renal blood vessels and renal tubules; the responses to angiotensin II are also affected by the functional state of the kidney and may be modified by the organ's content of renin.

Angiotensin II induces a constriction of the renal vascular bed, on both intravenous infusion into the whole animal, and on introduction into the renal artery (Assali & Westersten, 1961; Barer, 1961, Barac 1962). Spinal cord section and spinal anaesthesia reduces the renal vasoconstrictor effect of angiotensin II, indicating that it depends on an intact innervation of the kidney (McGiff, 1965).

In the rat and chicken, angiotensin II induces diuresis and natriuresis (Peters, 1963; Langford, 1964; Langford & Fallis, 1966). In other species, the rabbit, cat and dog, low concentrations of angiotensin II had an anti-diuretic and anti-natriuretic effect, higher concentrations were both diuretic and natriuretic (Gross & Turrian, 1960; Cort & Lichardus, 1963; Langford & Pickering, 1963, 1965). Normal human subjects responded with antidiuresis and sodium retention on infusion of angiotensin II (Nijensohn, 1957; Bock & Krecke, 1958; Laragh, Cannon, Bentzel, Sicinski & Meltzer, 1963).

Effect on adrenal cortex

The variations in renin content of the kidneys in different experimental conditions led to the conclusion that renin may stimulate aldosterone secretion (Gross, 1958, 1959). It was soon demonstrated that infusions of angiotensin II in subpressor and pressor concentrations increased aldosterone secretion and excretion in man (Genest, 1961; Genest, Biron, Koiw, Nowaczynski, Boucher & Chretien, 1961; Laragh, Angers, Kelly & Lieberman, 1960; Laragh, 1962), dog (Bartter, Casper, Delea & Slater, 1961; Davis, 1961) and sheep (Blair-West, Coghlan, Denton, Goding, Munro, Peterson & Wintour, 1962). Angiotensin II also stimulated the secretion of other adrenocortical hormones (cortisol, corticosterone), but higher concentrations were required than those which increased aldosterone secretion (Carpenter, Davis & Ayers, 1961a, b; Mulrow & Ganong, 1961a, b; Urquart, Davis & Higgins 1964; Ames, Borkowski, Sicinski & Laragh, 1965). In vitro, both perfused adrenals and adrenal slices have been found to secrete greater amounts of aldosterone in the presence of angiotensin II (Mulrow & Ganong, 1962; Kaplan & Bartter, 1962; Muller, 1962; Kaplan, 1963, 1965; Kumagai, Takeuchi, Ueda, Kotani & Yamamura, 1964)

h) Tachyphylaxis to angiotensin II

Tachyphylaxis to angiotensin II was first described by Page & Helmer (1940), measuring the blood pressure response of the anaesthetised dog. Onset of tachyphylaxis is much more rapid with large concentrations of angiotensin II, and cross tachyphylaxis exists between angiotensin II and renin (Page, McCubbin, Schwarz & Bumpus, 1957; Bock & Gross, 1961)

Most tissues that display a response to angiotensin II also exhibit tachyphylaxis; tachyphylaxis to angiotensin II occurs in the rabbit isolated heart (Beaulnes, 1963), adrenal medulla (Feldberg & Lewis, 1965), superior cervical ganglion (Lewis & Reit, 1965), rat isolated lung (Joubert, Tetreault & Biron, 1966). Innervated vas deferens and spleen (Benelli, della Bella & Gandini, 1964), kidney (Louis & Doyle, 1966) intestinal strips (Godfraind et al, 1966), uterus (Gross & Turrain, 1960) and many isolated arteries and veins from a variety of species, including man (Davingnon, Lorenz & Shepherd, 1965; Bohr & Uchida, 1967; Khairallah et al, 1966a). However, the latter authors reported an extreme difficulty in inducing tachyphylaxis to angiotensin II in the isolated thoracic aortae of the rabbit and guinea pig.

Liebau, Distler & Wolff (1965) first reported a reversal of tachyphylaxis to angiotensin II on a variety of mammalian isolated aortae, on incubation with noradrenaline; cocaine prevented the reversal of tachyphylaxis. However, Khairallah et al (1966)

could not repeat these results on the rabbit aorta, nor Palaic & Lemorvan (1967), on the guinea pig aorta. These later reports, however, have the disadvantage that they were performed on tissues exhibiting an atypical tachyphylaxis to angiotensin II, i.e. difficult to induce. Sakurai, Bunag & McCubbin (1967), using dog and cat isolated perfused mesenteric vessels, observed a reversal of angiotensin II tachyphylaxis after an infusion of noradrenaline, cocaine enhanced this reversal rather than preventing it; an infusion of 5-HT, however, did not reverse the tachyphylaxis. Further, although phentolamine did not affect initial responses to angiotensin II, it reduced them when they had been restored by noradrenaline.

Khairallah (1971) suggested that receptor saturation was the determining factor for angiotensin II tachyphylaxis; this suggestion is difficult to reconcile with the recent findings of Palaic & Lemorvan (1971) that during angiotensin II tachyphylaxis of the guinea pig isolated aorta there appear to be more available receptor sites for angiotensin II in comparison with controls, and that tachyphylaxis is more easily induced at a higher temperature.

i) Angiotensin II-antagonists

Many compounds have been examined as possible angiotensin II-antagonists, most have been found to be either ineffective or non-specific in their action. Today, most attention is focused on angiotensin II analogues.

The influence of anti-sympathetic and anti-parasympathetic drugs has been dealt with in the appropriate sections. Physiological antagonism to the vasoconstrictor action of angiotensin II has been reported by Thind (1973), who found, using the rabbit isolated aorta, that both cadmium and isoprenaline induced a relaxation of the aorta during responses to angiotensin II, the relaxation due to isoprenaline, but not that due to cadmium, was blocked in the presence of propranolol. Systemic vasodilators such as ATP, theophylline-ethylenediamine and papaverine (Bianchi, de Schaepdryver, de Vleeschhouwer & Preziosi, 1960) and bradykinin (Barer, 1961) reduce the pressor response to angiotensin II in whole animals. The possibility that this action of vasodilators is related to inhibition of phosphodiesterase has recently been investigated by Pettinger, Sheppard, Palkoski & Renyi (1973), who found, using ganglion-blocked anaesthetised rats, that directly acting vasodilatory drugs antagonised the pressor action of angiotensin II and noradrenaline equally, and further, that there was a quantitative correlation between phosphodiesterase inhibition and vasodilatation.

Lidoflazine antagonised the slow component of the contraction produced by angiotensin II in the guinea pig ileum (Godfraind, Kaba & Polster, 1966) however, on the rabbit isolated aorta and rat fundus, lidoflazine blocked responses to 5-HT, while not affecting those

to angiotensin II and noradrenaline (Turker & Kayaalp, 1967). Osajin has been reported to inhibit the action of angiotensin II on the guinea pig isolated ileum, while being ineffective against bradykinin (Gascon & Walaszek, 1966).

Day, Hall & Owen (1972) have reported that DDC inhibits the pressor response to angiotensin II in pithed rats, while not inhibiting responses to nerve stimulation, noradrenaline, 5-HT or vasopressin. Recently, Fleisch, Krzan & Titus (1973) have found that a related compound, dithiothreitol, selectively inhibits responses to angiotensin II of the rabbit isolated aorta, therefore, it appears that the direct constrictor response evoked by angiotensin II may involve an activation of sulphydryl groups which are inhibited by both DDC and dithiothreitol.

An isolated observation by Chong & Downing (1973) that responses of several isolated tissues (including rabbit aorta, rat fundus and guinea pig ileum) to angiotensin II are reduced by indomethacin suggests a possible involvement of prostaglandins in the response to angiotensin II.

The well known anti-hypertensive activity of diuretics (Earley & Orloff, 1964; Conway, 1965; Brest, Onesti, Seller, Ramirez, Heider & Moyer, 1965; Dikshit, Nath & Sikka, 1965; Hutcheon, 1967) has led to their examination as possible anti-angiotensin II compounds.

Pretreatment with thiazide diuretics reduced the pressor activity of both angiotensin II and noradrenaline in dogs (Bock & Gross, 1960; Gillenwater, Scott & Frohlich, 1962) but acute administration of chlorothiazide had no effect on the angiotensin II response in man (Silah, Jones, Bashour & Kaplan, 1965). However, Zech, Sassard, Pozet & Traeger (1970), using either hydrochlorthiazide or ethacrynic acid on hypertensive patients, observed a potassium deficit with hypokalaemia and significantly reduced the response to angiotensin II; high doses of potassium added during diuretic treatment maintained a negative sodium balance and a positive potassium balance restored the kalaemia and returned the pressor response of angiotensin II to control values.

Perez-Olea, Quevedo, Munoz & Illanes (1969) found that pretreatment with frusemide significantly reduced the response to angiotensin II in pithed rabbits, while not affecting the response to tyramine and noradrenaline. Finch (1971) found that treatment of rats orally with frusemide reduced their hypertension to normotensive levels, while not affecting responses to nerve stimulation or noradrenaline in the pithed animals. This suggests the involvement of a humoral factor in hypertension, which may or may not involve angiotensin II.

In vitro, the situation is equally confused; in rabbit isolated aortae, Napodano, Caliva, Lyons, De Simone & Lyons, (1962) report no angiotensin II-inhibiting action of benzydroflumethiazide, whereas, using the rat portal vein, Blair-West, McKinley & McKenzie (1972) reported a non-specific blocking action of frusemide on a variety of agonists.

SECTION B Physiology and Pharmacology of vascular smooth muscle

a) Structure of the vascular wall

The arterial wall consists of the intimal (inner) medial and adventitial coats, the medial layer being the main site of accumulation of smooth muscle cells; in fact, smooth muscle cells are the only cell type found within the aortic media of mammals (Bierring & Kobayashi, 1964; Pease & Paul, 1960; Cliff, 1967) and are responsible for the production of collagen and elastin. Vasa vasorum supply the medial coat of thicker arteries with essential metabolites (Wolinsky & Glagov, 1967).

The orientation of smooth muscle in the artery is usually spiral or circular, as opposed to the usually longitudinal arrangement in veins. In the rabbit thoracic aorta, Elchlepp & Furchgott (1955) found an essentially circular orientation of smooth muscle, and practically no longitudinally orientated smooth muscle. This orientation is important in two respects; when the artery is cut spirally it provides a sheet of longitudinally orientated smooth muscle fibres, and the direction of cutting of the spiral does not alter the responses, as opposed to the case with ox mesenteric arteries reported by Hausler (1933).

b) Contractile and regulatory proteins

The mechanical events responsible for the contraction of vascular smooth muscle are associated with it's contractile proteins. These proteins not only develop the mechanical force responsible for the contraction, but also act as the enzyme that catalyses the release of energy by which this force is developed (Bohr, 1973).

Until comparatively recently, the nature of contraction in smooth muscle has been in doubt; especially with regard to the structural and functional differences between smooth and striated muscle. However, the results of Kelly & Rice (1968) using gizzard and Nonomura (1968) using guinea pig taenia coli show two types of filaments; thick and thin, their dimensions corresponding to those in skeletal muscle. However, Schirmer (1965) found that, using hybrids made from skeletal muscle actin and myosin and smooth muscle actin and myosin, the ATP-ase activity depended on the source of the myosin. This correlates well with the observations of Barany (1967), showing that a direct parallel exists between the maximum velocity of shortening of a muscle and the ATP-ase activity of it's actin-activated myosin.

Somlyo, Devine, Somlyo & Rice (1973) found both thick and thin filaments in rabbit portal vein. They also observed filaments of intermediate thickness;

these were suggested by Ruegg (1973) to represent tropomyosin B (see below). Contraction of vascular smooth muscle most probably is effected by some version of the Huxley sliding filament mechanism (Huxley, 1957).

Ebashi & Ebashi (1964) first demonstrated that a protein complex, troponin-tropomyosin, from skeletal muscle inhibits the ATP-ase activity of actomyosin; this inhibition is depressed by free ionic calcium. Contraction occurs when an increase in the ionic calcium concentration reduces troponin-tropomyosin inhibition and allows activation of the actomyosin ATP-ase. In fact, native tropomyosin (troponin-tropomyosin-β-complex) is now widely accepted as the system providing calcium-sensitivity to the contractile system (Ebashi, Endo & Ohtsuki, 1969). Troponin is the calcium-receptive protein, and is bound to tropomyosin; the latter in turn is associated with F-actin filament, i.e. the thin filament is a complex of three proteins; troponin, tropomyosin and F-actin. Troponin has been isolated from chicken gizzard and dog heart, as a complex with tropomyosin, in a similar amount to that in skeletal muscle (Ebashi, Iwakura, Nakajima, Nakamura & Chi, 1966).

c) Biochemistry

Carbohydrates are the major, but not immediate, source of energy in vascular smooth muscle as indicated by a respiratory quotient close to one (Kosan & Burton, 1966; Wertheimer & Ben-Tor, 1960). It is probable

that, as in skeletal muscle, preformed glycogen is utilised preferentially by vascular smooth muscle, as there is no stimulation of respiration on addition of glucose to the medium containing isolated vascular segments, including rabbit aorta (Chattopadhyay, 1961; Henry, Gras & Piquard, 1967). Glycogen can be synthesized by vascular smooth muscle, in vitro, from glucose added to the medium; this synthesis, in the rat aorta, is increased by noradrenaline (Wertheimer & Ben-Tor, 1961).

As in the case of other smooth muscle, and vascular smooth muscle, the immediate source of energy for contraction of actomyosin of vascular smooth muscle appears to come from the splitting of the terminal "high energy" phosphate of endogenous ATP, with the formation of ADP. Regeneration of ATP is by either oxidative phosphorylation or glycolysis (aerobic or anaerobic). In bovine mesenteric arteries the Pasteur effect, inhibition of lactic acid production by oxygen, is clearly seen (Lundholm & Mohme-Lundholm, 1960, 1962).

Furchgott (1966) described experiments performed on glycogen-depleted aortic strips; these had been exposed to anoxia in a substrate-free medium for one hour. There was only a minute contraction to adrenaline, even at 10^{-6} g/ml. However, on addition of glucose a contraction ensued in response to adrenaline which was 75% as strong as the contraction obtained under oxygen in the presence of glucose.

However, the substrate-dependence of contractions of arteries depend on two factors: (1) the nature of the agonist, and (2) the nature of the substrate (see below). Altura & Altura (1970), using rabbit isolated aortae, found that the tissue requires a continuous supply of exogenous glucose in order to maintain a contraction to angiotensin II, histamine and barium; after two hours incubation in glucose-free aerobic medium, there is a 40% reduction in the response to angiotensin II, and after four hours there is no response at all. However, responses to KCl and catecholamines do not appear to be affected over this period of time. The authors suggested that the lack of response to angiotensin II, histamine and barium was due either to a failure of active transport mechanisms induced by substrate depletion, or a loss of integrity of their receptor systems.

The nature of the carbohydrate used as the exogenous substrate appears to be very important in vascular smooth muscle. Under aerobic conditions, Coe, Detar & Bohr (1968) exposed rabbit aortic strips to high concentrations of adrenaline, in substrate-free medium, to deplete endogenous substrate stores. They found that addition of either glucose or mannose to the medium returned the response to adrenaline, whereas fructose and xylose were ineffective, suggesting a selective sugar-transport mechanism. Earlier work by Wertheimer & Ben-Tor (1962), using the rat aorta, had suggested a similar conclusion, as insulin-stimulated uptake of glucose, galactose, xylose and arabinose, but not of fructose.

d) Ions

Calcium

It was first demonstrated by Ringer (1883) that isolated muscles would not contract if exposed to a medium deficient in calcium. This was substantiated by Heilbrunn & Wiercinski (1947), who observed, using an intracellular microinjection technique, that calcium was the only substance which on injection into the cell would induce a contraction. Niedergerke (1955) demohstrated that the magnitude of tension was directly related to the intracellular calcium concentration.

In a recent brief review, Bohr (1973) has described the possible intracellular sites of storage of calcium in vascular smooth muscle, and noted that "activator" calcium (i.e. calcium to activate the actomyosin ATP-ase and hence release energy for contraction) may come from intracellular and/or extracellular sources, depending on the agonist. Three main organelles have been proposed as sites for storage of intracellular calcium; the sarcoplasmic reticulum, mitochondria and the cell membrane. Devine, Somlyo & Somlyo (1973) have demonstrated that the sarcoplasmic reticulum occupies an appreciable part of the vascular smooth muscle cell, ranging from over 5% of the volume in the aorta and the main pulmonary artery of the rabbit to approximately 2% in the portal anterior mesenteric vein and the mesenteric artery. They further observed that smooth muscle containing the larger

amounts of sarcoplasmic reticulum maintains it's contraction better in a calcium-free environment than does muscle with relatively little intracellular sarcoplasmic reticulum. Also, mitochondria of the vascular smooth muscle cell accumulate barium and strontium, and, therefore, are possible sequestration sites for calcium.

It is apparent that different agonists may utilise a different proportion of intra- and extra-cellular calcium ion to induce a contraction. Waugh (1962) found that the contraction of canine isolated arteries (perfused) by a depolarising solution of K_2SO_4 was prevented in a calcium free, EDTA-containing medium; the high-potassium depolarising solution appeared to be increasing the influx of extracellular calcium. Briggs (1962) made a similar observation using rabbit isolated aortic strips. He found that potassium-induced contraction was initiated by an influx or release of calcium into the vicinity of the contractile elements of the aorta, and that the maintenance of the contraction was dependent on a sustained level of ionised calcium in this region. The results obtained by Hudgins & Weiss (1968) demonstrated a difference between the extracellular calcium dependency of noradrenaline and potassium ions; using the rabbit aorta they found that in a calcium-free EDTA-medium, responses to potassium-induced depolarisation were almost abolished, whereas responses to noradrenaline were reduced to 28% of controls. Noradrenaline appears to utilise both intra and extracellular calcium by adrenaline. They found

a dual response to adrenaline, a fast component followed by a slow component; in calcium-free solutions the fast component was initially potentiated because membrane excitability was increased, but the slow component was depressed, in high calcium solution the converse was true. It appears that the initial fast component of the response to adrenaline is due to a release of calcium from intracellular storage sites, whereas the slow sustained component of the response is due to an influx of extracellular calcium.

Kalsner, Nickerson & Boyd (1970) attempted to selectively block transmembrane calcium movements by the use of the metabolic inhibitor SKF 525A. They found, using the rabbit isolated aorta, that contractions to KCl were blocked in the presence of SKF 525A, responses to noradrenaline were reduced after 4 hr contact with the antagonist, but responses to angiotensin II were not significantly affected. Other compounds which effectively decrease membrane permeability to calcium have been used to study the source of activator calcium; they include cinnarizine (Godfraind& Kaba, 1969), verapamil (Peiper, Griebel & Wende, 1971; Haeusler, 1972 Golenhofen & Lammel, 1972) and lanthanum (van Breemen, Farinas, Gerba & McNaughton, 1972). Vascular smooth muscle incubating in a calcium-free high-potassium depolarising solution can be caused to contract by the addition of low calcium concentrations to the muscle bath (Carrier & Jurevics, 1972). This response presumably occurs because the added calcium passes

through the membrane which has been made highly permeable by the K_2SO_4-induced depolarisation. Various calcium blocking agents greatly reduce, or eliminate, this response. These agents are much less effective in eliminating the response produced by agonists such as adrenaline, angiotensin II or histamine, suggesting that the contractile responses initiated by these agonists are less dependent on the passage of extracellular calcium through the cell membrane than is the contractile response initiated by potassium-induced depolarisation.

van Breeman et al (1972) demonstrated that lanthanum blocks the passage of calcium ions across the cell membrane of vascular smooth muscle, and that it replaces all calcium bound to extracellular structures. They found that there were parallel rates of increase in tension and in intracellular calcium content in response to activation by potassium-induced depolarisation or by lithium substitution for sodium. However, noradrenaline induced a maximum increase in tension with no measurable increase in intracellular calcium concentration, indicating that noradrenaline uses mainly intracellular calcium. When the calcium pool was discharged by noradrenaline, histamine or angiotensin II after the muscle has been in a calcium-free lanthanum containing medium for 30 min., a single contraction occurs, but the muscle will not subsequently respond to stimulation by any of the three agonists. This indicates: that all spasmogens utilise a final common pathway, probably release of calcium and combination with acto-myosin ATP-ase; and also that this calcium store needs replenishment from extracellular calcium.

A further discussion of the involvement of calcium in the response to angiotensin II is given in Section A (p. 29)

Sodium

Studies with radioactive sodium and potassium ions on the dog carotid artery (Garrahan, Villamil & Zadunaisky, 1965) and rat aorta (Hagemeijer, Rorive & Schoffeniels, 1965) indicate a very high permeability of vascular smooth muscle to sodium. The kinetics of sodium efflux suggest the presence of three predominant compartments: the first, with a half-life of 2.5 to 5 min. is probably intracellular; the third compartment, with the slowest exchange rate of 40 to 70 min. contains less than 2% of the total sodium in either arterial preparation.

It has been shown by direct observation that the membrane potential of vascular smooth muscle is an appropriate function of the $(K)^{+}_{I}$: $(K)^{+}_{O}$ ratio (Nakajima & Horn, 1967); however, in smooth muscle, to a much greater extent than in cardiac and skeletal muscle, the membrane potential is influenced by the transmembrane concentration gradient of sodium. For this reason, it may be anticipated that an elevation in the $(Na)^{+}_{O}$: $(Na)^{+}_{I}$ ratio might significantly decrease resting membrane potential and, therefore, increase excitability. However, vascular smooth muscle has physiologically important non-electrical activation pathways; these are dealt with later.

The higher electrochemical potential of extracellular sodium suggests that an active process maintains the transmembrane sodium gradient. There is now considerable evidence for the existence of an active sodium pump in vascular smooth muscle (Barr, Headings & Bohr, 1962; Daniel, 1965b; Garrahan et al, 1965; Hagemeijer, 1965; Vidlakova & Kleinzeller, 1963; Gulati & Jones, 1971; Gulati, 1973). The extrusion of sodium and uptake of potassium against their electrochemical gradients appear to be mediated by a system which has the properties of a Na,K-dependent ATP-ase (Skou, 1965; Thomas, 1972). The sodium pump of vascular smooth muscle is inhibited by ouabain and dinitrophenol (Garrahan et al, 1965; Hagemeijer et al, 1965) It is also markedly inhibited when isolated arteries are incubated in potassium-free medium (Garrahan et al, 1965; Thomas, 1972) and Daniel (1965b), has observed a marked inhibition of the sodium pump of the rabbit isolated aorta on prolonged incubation in calcium-free, EDTA-containing medium.

A great deal of evidence has recently been accumulated which suggests that besides the Na-K pump on vascular smooth muscle membrane, there is also calcium-sodium linked transport. Baker, Blaustein, Hodgkin & Steinhardt (1969) demonstrated that, in the membrane of the squid axon, there exists an active transmembrane electrolyte pump which couples sodium efflux to calcium influx. The existence of such a

pump in vascular smooth muscle would require that either incubating the tissue in a sodium free environment or increasing the $Na^+_I : Na^+_O$ ratio of the tissue (e.g. by ouabain) would increase the reactivity of the tissue, presumably by a stimulation of the Na^+/Ca^+ pump and result in an increase in the activator calcium concentration. Briggs & Shibita (1966) have observed that ouabain-induced contractions of the rabbit isolated aorta require calcium in the bathing medium, and are associated with an increased uptake of extracellular calcium. However, Broekaert & Godfraind (1973) have shown that responses of rabbit isolated blood vessels to ouabain were abolished in the presence of phentolamine, whereas those of the guinea pig aorta were not. Similarly, a potentiation of the response to noradrenaline in the presence of ouabain, seen by Bohr, Seidel & Sobieski (1969), using rabbit aorta and mesenteric artery, may have been due to a mechanism other than a direct action on vascular smooth muscle.

George & Leach (1973), using the perfused rat mesentery preparation, have observed a calcium-dependent antagonism of the responses to tyramine and octopamine by ouabain, and a calcium-dependent potentiation of the responses to noradrenaline and metaraminol. This obviously cocaine-like action of ouabain must be taken into account before interpretations of potentiations are based on the possible existence of a sodium-calcium pump.

Swanson & Ullis (1966) have observed a respiratory-stimulating action of ouabain on isolated slices of rat cerebral cortex. This was calcium-dependent, as in calcium-free medium ouabain induced a depression rather than a potentiation of cellular respiration. Therefore, potentiation of responses to agonists by ouabain, and the calcium-dependency of this process, may indicate a stimulation of respiration rather than an indirect action of ouabain via the adrenergic neurone.

Recently, biochemical evidence has been presented by Limas & Cohn (1973) to suggest the presence of a calcium-sodium ATP-ase in canine aorta, and the authors present further evidence that angiotensin II induces a contraction by stimulating this pump. More evidence is obviously required to decide whether this is a direct or an indirect action of angiotensin II, i.e. mediated via other ion movements. Also, the results obtained by Bohr et al (1969) exposing isolated rabbit vascular tissue to sodium free medium enhanced the response to catecholamines; this procedure would be expected to cause a stimulation of the sodium-calcium pump.

Friedman, Jamieson & Friedman (1959), first postulated that the sodium gradient of the vascular smooth muscle cell, i.e. the ratio of $(Na)^{+}_{I} : (Na)^{+}_{O}$ is the primary determinant of vascular smooth muscle tone. The results of the work described above using

ouabain on isolated vascular smooth muscle suggest that an increase of the $(Na)_I^+ : (Na)_O^+$ ratio increases the reactivity of vascular smooth muscle. Alteration of the sodium concentration of the medium bathing vascular smooth muscle has resulted in a series of somewhat conflicting reports. Using the rabbit isolated aorta, Bohr, Brodie & Cheu (1958) reported an increased reactivity of the tissue to catecholamines following a 15 min. incubation in a low sodium medium; However, Yamabashi & Hamilton (1959), using canine isolated aorta found that responses to noradrenaline varied directly with external sodium concentration, with a potentiation at high sodium and a reduction at low levels. Dodd & Daniel (1960), using the rabbit isolated aorta, reported a progressive decrease in responsiveness of the tissue during incubation in sodium-free medium. Napodano, Caliva, Lyons, DeSimone & Lyons (1962), however, using angiotensin II as the sole agonist, have observed an increased response of rabbit aortic strips when the external sodium was reduced by 25% during contact of the agonist with the tissue; conversely, when the external sodium was increased during contact of the drug with the tissue, the size of the response was reduced. Similarly, Bohr, Seidel & Sobieski (1969) observed a potentiation of the response to noradrenaline when rabbit isolated aortae were exposed to sodium free medium; but they only allowed a 30 sec. equilibration period before drug-tissue contact. From the observations of Nash, Luchka & Jhamandas (1966) it

appears that tissue equilibration period with sodium-deficient medium is the critical factor; using rabbit isolated aorta, they found that when an 18 min. equilibration period was allowed after reducing the sodium content, no clear potentiation was observed, but when only one minute was allowed for equilibration a definite potentiation was observed. They made the interesting corollary observation that this latter potentiation was found regardless of the level of external calcium; i.e. the potentiation was related directly to the $(Na)_I^+ : (Na)_O^+$ ratio, and did not appear to involve any sodium-calcium competition at the cell membrane.

Recently, Harris & Palmer (1972) have found that isolated perfused vascular segments of the rabbit show an increased responsiveness to a variety of agonists (noradrenaline, histamine, nerve stimulation and angiotensin II) when the medium sodium was increased by 5%. Isotope-exchange studies with radioactive sodium indicated that this small increase in environmental sodium concentration caused an increased sodium ion content of 20% in the arterial wall, i.e. a marked increase in the $(Na)_I^+ : (Na)_O^+$ ratio, even though the environmental sodium was raised. However, this appears to be a rather simplified picture, as Blair-West, Harding & McKenzie (1968), using the rabbit isolated ear, have observed an agonist-selective effect of sodium, in that low sodium perfusates inhibited responses to angiotensin II by 50% to 60% while

responses to noradrenaline were little affected, whereas high sodium perfusates also inhibited responses to angiotensin II and responses to noradrenaline were transiently inhibited. As these alterations in the sodium content were large (20% to 25%), and the sodium-sensitivity of angiotensin II has been observed by many authors (see Section A), the ratio of $(Na)_I$: $(Na)_O$ is difficult to interpret.

Sodium may also have a direct effect on smooth muscle contractile proteins. Shibita, Yamagami & Akagami (1971) have observed an enhancement of the superprecipitation of bovine carotid myosin B when the Na^+ : K^+ ratio in the incubating medium is raised. The action on contractile proteins may also be indirect, but still intracellular; Fitzpatrick, Landon, Debbas & Heurwitz (1972) have observed a calcium-magnesium-ATP-ase in a microsomal cell fraction derived from the intimal-medial layer of rabbit aorta, this cell fraction takes up calcium quantitatively.

There is a Na^+/Ca^{2+} pump at the level of the smooth muscle cell membrane. As there are intracellular storage sites of calcium, there is possibly an exchange between calcium and sodium at these sites. Hence, an increased intracellular free sodium level would cause an increased intracellular free calcium level.

Potassium

The relationship between $(K)_O^+ : (K)_I^+$ is even more complicated, as regards contractility, than the relationship between $(Na)_O^+ : (Na)_I^+$; both raising and lowering external potassium has been reported to induce a contraction of isolated arteries (see Chap. V). Contractions induced by potassium on the rabbit aorta are associated with calcium influx, as they are totally abolished in calcium free Krebs (Waugh, 1962); this contraction is associated with a depolarisation of the smooth muscle cell membrane, but as Haeusler (1973) has found, treatment of the rabbit main pulmonary artery with verapamil had no effect on high potassium-induced depolarisation, but totally blocked the contractile response. Although the high potassium-induced contraction is totally dependent on external calcium, potassium-free-induced contraction shows no calcium dependency (Distler, Grobecker, Kreye & Lazar, 1973).

e) Excitation-contraction coupling

Somlyo & Somlyo (1968), in the first part of their review of the physiology of vascular smooth muscle, have described two possible mechanisms for the transduction of the combination of drug with it's receptor into a contraction of smooth muscle; they termed these mechanisms electromechanical coupling and pharmacomechanical coupling. The former mechanism obviously suggests a coupling between membrane action potentials and smooth muscle contraction, such as the contractions of rat portal-mesenteric veins associated with

spontaneous action potentials. However, there is uncertainty as to whether the action potential is inducing calcium influx which directly activates the myofibrils or if it is discharging calcium from an intracellular storage site in spike-generating vascular smooth muscle. It seems unlikely that a calcium current is responsible for spike activity and that the amount of calcium charge is sufficient to activate contraction, as Axelsson, Wahlstrom, Johansson & Jonsson (1967) have found that calcium-free solutions, after an initial period of enhanced spike-activity, depolarise and abolish the action potentials of rat portal veins; contraction amplitude decreased before the spike amplitude. Also, Johansson, Jonsson, Axelsson & Wahlstrom (1967) found that noradrenaline could initiate spike activity without inducing contraction when the rat portal vein was incubated in calcium-free medium. Somlyo & Somlyo (1968) have observed the converse case; action potentials can be abolished, without depolarisation, in spike-generating vascular smooth muscle, while the contractile effects of drugs persist.

The evidence for the existence of pharmacomechanical coupling, i.e. the action of compounds on the contractile system independent of the membrane potential and action potentials, has been presented by Somlyo & Somlyo (1970) in part two of their review of vascular smooth muscle. It is as follows :-

(i) The same blocking and potentiating agents are effective in polarised and depolarised smooth muscle (Evans, Schild & Thesleff, 1958; Somlyo & Somlyo, 1968; Waugh, 1962)

(ii) Depolarisation by drugs may be less but the maximal contraction greater than the respective effects of high potassium solutions (Somlyo & Somlyo, 1968; Somlyo, Vinall & Somlyo, 1969)

(iii) The differences in the maximal contractile effects are maintained after depolarisation (Somlyo & Somlyo, 1968, 1970)

(iv) Relaxing agents can relax polarised smooth muscles without evidence of hyperpolarisation (Diamond & Marshall, 1969)

(v) Dissociation of the electrical and the contractile effects of drugs is readily seen in polarised smooth muscle (Cuthbert & Sutter, 1965; Somlyo & Somlyo, 1968, 1970).

Somlyo & Somlyo (1970) suggest that an increased membrane permeability to calcium is produced by several, and perhaps all, excitatory drugs and that this effect is the major mechanism of near-maximal and maximal contractions. However, responses to various agonists vary as to their dependency on extracellular calcium, and it seems reasonable that a

proportion of agonists may act by a release of intracellularly-bound calcium, as found for angiotensin II acting on the rabbit aorta by Angles d'Auriac et al (1972).

f) Adrenergic pharmacology

The adrenergic pharmacology of smooth muscle is an extremely large topic, so that only a general discussion will be given here. Specific details are dealt with in the appropriate chapter.

Bevan, Gillespie & Johansson (1969) summarised the evidence relating to the sympathetic nervous system in vascular smooth muscle. They note that the sites of transmitter storage and release in blood vessels are almost invariably found only at the junction between the tunica adventitia and the tunica media, therefore, there is a separation between the neural elements and the bulk of the muscular elements. Noradrenaline is synthesized and stored prior to release in the plexus at the adventitio-medial junction; when released it diffuses inwards towards the intima and outwards towards the adventitia. Maxwell, Eckhardt & Wastilla (1968) found that 78% of the endogenous noradrenaline of rabbit thoracic aorta is found in the adventitia, and in this region resides 75% to 90% of the capacity of the tissue to bind exogenous noradrenaline, a cocaine-sensitive binding. Removal of the adventitia drastically reduced the responsiveness of aortae to tyramine.

Inactivation of released transmitter may occur by two mechanisms in vascular smooth muscle: neuronal uptake (Uptake I) and extraneuronal uptake (Uptake 2), (Iversen, 1967). Extraneuronal uptake of catecholamines in the vascular wall may occur into muscular and fibrous elements, the former being inhibited by steroids (Iversen & Salt, 1970) and the latter being inhibited by oxytetracycline (Powis, 1973). Neural uptake of noradrenaline into rabbit isolated aortae has been shown to be inhibited by guanethidine and cocaine (Maxwell, Daniel, Sheppard & Zimmerman, 1962) Bevan et al (1969) state that in rabbit aorta and pulmonary artery, spread of excitation from outer to inner muscles seems to depend on diffusion of the transmitter through the extracellular space; rather than intermuscular conduction. These vessels respond slowly to drugs and exhibit little spontaneous contractile activity. Mammalian hepatic-portal veins exhibit spontaneous rhythmic activity, originating from pacemaker sites; there appear to be two mechanisms for modulation of activity; transmitter modification of pacemaker activity, and direct action by diffused transmitter.

g) Hypertensive vessels

The major question regarding the functional properties of hypertensive vessels is whether or not they are hyper-reactive to electrical stimuli. Somlyo & Somlyo (1970) found that the majority of studies are consistent with a relatively non-specific

hyper-reactivity of blood vessels. The secondary question regarding this abnormal state of vascular function is whether or not there is a morphological and/or functional change of the vessel.

Increased wall thickness and decreased lumen may lead to an apparent increase, due to mechanical factors, in the responsiveness of blood vessels to fixed concentrations of vasoconstrictors (Barany, 1963; Baum & Shropshire, 1967; Dahl, Heine & Tassinari, 1964; Davis & Landau, 1966; Demura, Fukuchi, Takahashi & Goto, 1965; Gordon & Nogueira, 1962; Hinke, 1965; McGregor & Smirk,1968).
An increased wall thickness due to stimulation of the protein synthesis mechanisms in the vascular smooth muscle cell has recently become feasible since the observations of Khairallah, Robertson & Davila, (1972) that angiotensin II, a possible factor in some forms of hypertension (Haber, 1969; Lee, 1969; Peart, 1969) does stimulate protein synthesis. Friedman (1973) has observed that, although DOCA-hypertension of the rat is characterised by an increased surface area and volume of the smooth muscle cell, without shortening, thus encroaching on the area of the lumen, post-DOCA (sustained) hypertension echibits no cell hypertrophy; he concluded that smooth muscle hypertrophy is not a necessary condition of the sustained hypertensive state, in which the extra load is carried not by the cells, but by a considerably increased paracellular matrix and collagen net.

Tobian & Binion (1964), Tobian (1960) and Jones, Feigl & Peterson (1964) have reported increased water and electrolyte contents of the arterial wall in association with experimental hypertension of renal and steroid origin. Hinke (1966), using rat isolated artery strips, found that the dose-response curve of calcium-depleted vessels to added calcium was shifted to the left in hypertensive animals (DOCA saline), which appears to indicate either an increased membrane permeability to calcium, or a decreased activity of the relaxing system of vascular smooth muscle in hypertensive animals. Using isolated aortae taken from spontaneously hypertensive rats, Jones (1973) found that they exhibited a significantly decreased ability to accumulate potassium and extrude sodium in comparison with control tissues. He also observed that in both groups of rats both angiotensin II and noradrenaline increased the rate of potassium exchange.

It seems reasonable to propose that both morphological and functional changes occur in the vessel wall of hypertensive animals, and that these factors may contribute to the maintenance of the hypertensive state.

AIMS OF THESIS

The initial aim of this investigation was to elucidate the mechanism of interaction of angiotensin II with endogenous and exogenous noradrenaline. As the work progressed, it became evident that, in the case of the rabbit isolated aorta, the potentiating action of angiotensin II was not confined to noradrenaline, but took the form of a non-specific sensitisation. The latter part of the thesis is devoted to an investigation of the possible mechanism of this interaction and, in particular, the involvement of ions. Once a hypothesis had been developed, it was examined for its involvement in the actual constrictor action of angiotensin II in the aorta and rat colon, to determine whether the direct and indirect actions of angiotensin II were based on a common mechanism of action.

The actions of angiotensin II which have been discovered and examined during the course of this investigation are discussed, and related to vascular physiology, particularly at the ionic level.

METHODS

METHODS

ISOLATED VASCULAR PREPARATIONS

(a) Rabbit ear central artery

Rabbits were stunned by a blow on the back of the neck and bled out. Both ears were removed and polyethylene cannulae inserted into the central artery of each ear as near the base of the ear as possible. The blood within the vascular bed was flushed out with the perfusion fluid and the ears were stored in a beaker of perfusion fluid at 4°C. This artery was used, if required, if storage had not exceeded 4 hours. The artery removed from the second ear was dissected free of surrounding tissues and a length of 4-5cm taken for perfusion. These arteries were set up for perfusion as described by de la Lande & Rand (1965), except that the artery was suspended in air instead of in an organ-bath containing the perfusion solution. In order to obtain access of drugs to the outer surface of the artery only, some arteries were double-cannulated and suspended in a horizontal organ bath, a procedure previously described by de la Lande & Rand (1965). The preparation was perfused with Kreb's bicarbonate solution containing (g/l): Na Cl 7.7, K Cl 0.34, $CaCl_2$ 0.3, KH_2PO_4 0.16, $MgSO_4$ 0.29, $NaHCO_3$ 2.1 and glucose 1.0. The fluid was maintained at 37°C and continuously gassed with 95% oxygen and 5% carbon dioxide. Perfusion pressures were recorded by using a Bell & Howell Type 4-327-L221 pressure transducer connected to a Devices M2 or M4 electronic recorder. Perfusion rate 4·0ml/min.

Drugs were dissolved in saline and injected into the arterial cannula in a volume not exceeding 0.1ml, or were added to the reservoir containing the perfusion fluid.

The vascular sympathetic nerves were stimulated by threading the artery through bipolar platinum electrodes (Burn & Rand, 1960) and delivering rectangular pulses from an electronic stimulator (Scientific & Research Instruments). In general, pulses of supramaximal strength (30 volts) of 1 millisecond duration and frequencies of 1-50Hz were used. Stimulation was usually applied for periods of 40 seconds in every 4 minutes.

(b) Rabbit isolated perfused whole ear

The animal was killed, and the central artery of the rabbit's ear cannulated as above. The whole ear was suspended in a warm chamber at 37°C. Stimulation procedures (electrical and drug) were as described above.

(c) Rabbit isolated perfused mesenteric bed

The animal was killed as above. The superior mesenteric artery was cannulated as described for the rat by McGregor (1965). The mesenteric vascular bed was gently separated from the viscera, and suspended in a petri dish containing Krebs solution. The external solution was supplied from a heated reservoir to prevent prolonged contact of drugs added intra-arterially with the outer surface of the blood vessels. Stimulation parameters were as described for the previous preparations.

(d) Rabbit isolated aortic strip

Rabbits of either sex, weighing between 2.0 and 3.5 kg were stunned and exsanguinated. The thoracic aorta was rapidly removed, placed in ice-cold Krebs solution, cleared of fat and cut into a close spiral. The spirally-cut thoracic aorta (average length 4.0cm) was suspended in Krebs bicarbonate solution (Regoli & Vane, 1967), under 2g tension, and gassed with 95% oxygen, 5% carbon dioxide. All experiments were conducted at 37°C, in a 30ml drainout muscle chamber; 2 hr were allowed for equilibrium and full relaxation of the strips before drugs were added. Unless otherwise stated, agonist drugs were allowed to evince their maximum contraction at a given concentration before being washed out of the bath. There was a 15 min. relaxation period between washout of one dose and addition of the next. Contractions were recorded on a smoked drum by means of a frontal writing lever exerting a load of 2.0g and having a magnification of 12.5:1.

Pretreatment with reserpine

Rabbits were reserpinised by the method of Hudgins & Fleming (1966), who found that an intravenous dose of 0.3 mg/kg for three days had a maximum effect on the shift of the dose/response curve of the isolated aorta, to the left for the directly-acting noradrenaline and to the right for the indirectly-acting tyramine.

Rat isolated colon

Male rats weighing 180-250g were stunned by a blow on the head and killed by cervical dislocation. Segments of ascending colon (from the region exhibiting diagonal striations immediately adjacent to the caecum) were removed, and a piece 15-20mm in length suspended in Kreb's solution in a 25ml organ bath at 37°C gassed with 5% carbon dioxide and 95% oxygen. Longitudinal contractions were recorded on a smoked drum by means of a frontal writing lever exerting a load of 1.5-2.0g and having a magnification of 10.

Normal and modified bathing media

i) Normal Krebs

Composition: Na Cl 6.9g/l, K Cl 0.35 g/l, $CaCl_2 2H_2O$ 0.37 g/l, K_2PO_4 0.16 g/l, $MgSO_4.H_2O$ 0.29 g/l, glucose 1.0 g/l and ~~Mc~~NaHCO_3 2.1 g/l.

ii) Ca^{2+}-free Krebs

Composition: this was made as for normal Krebs except the calcium was not added. No osmotic adjustment was made for the absence of calcium.

iii) Ca^{2+}-rich Krebs

Composition: 100% more $Ca Cl_2 2H_2O$ was added to the Krebs. No correction was made for change in osmolarity.

(iv) Na^+-rich Krebs

Composition: either 20% or 5% more sodium chloride was added to normal Krebs. Again, no correction was made for change in osmolarity.

v) K^+-free Krebs

K Cl was replaced by Na Cl, and K_2HPO_4 by Na_2HPO_4 in equimolar quantities. Composition: Na Cl 7.17 g/l, Ca Cl_2 $2H_2O$ 0.37 g/l, Na_2HPO_4 0.18 g/l, $MgSO_4 7H_2O$ 0.29 g/l, glucose 1.0 g/l and Na_2HCO_3 2.1 g/l.

In each case, the tissue was allowed to equilibrate in the modified Krebs for 2 hr, the tissue being washed every 15 min., before addition of drugs.

Measurement of radioactivity

Aortae were removed from exsanguinated rabbits, placed in ice-cold Krebs solution, cleared of fat and sliced into the required number of rings. Each ring was blotted dry, weighed and replaced in ice-cold Krebs solution. Drugs to be tested were added, and the aortic rings incubated at 37°C for 10 min, at the end of this period ^{14}C-d,l-noradrenaline (6.7×10^{-8} g/ml) was added. At the required time, aortae were removed from the incubation mixture, rapidly cooled, placed in sintered glass funnels and washed with two 10 ml aliquots of ice-cold Krebs. The aortae were then digested overnight in a scintilation vial, containing 1 ml of "Protosol" (New England Nuclear Corporation) and 0.25 ml of water, at 55°C. 5 ml of scintillation fluor * were added to each vial, the whole mixed in a "Whirlimix", and counted in a Beckman LS-230 liquid scintillation counter.

After the initial 1 min. count, samples were left overnight and then recounted, each for a 10 min period, as an initial output of photoradiation occurs when fluor is mixed with "protosol". Results were plotted as uptake in cpm per mg. of wet tissue. Throughout this section on radioactivity measurements, all Krebs solutions referred to contained, per litre, 20mg EDTA, 20mg ascorbic acid and 2.0mg pargyline to prevent breakdown of noradrenaline by heavy metals, oxidation and monoamine oxidase respectively (Horn, Coyle & Snyder, 1971).

The pithed rat preparation

Male Wistar rats weighing 190-300g were anaesthetised with pentobarbitone ("Nembutal" - 60mg/kg intraperitoneally). The trachea was cannulated and the animal pithed via the right orbit by the method of Shipley & Tilden (1947), using a steel pithing rod, 1.5mm in diameter, prepared as described by Gillespie & Muir (1967), for the stimulation of sympathetic outflow. Immediately after pithing, positive pressure artificial respiration was commenced using a Palmer small animal respirator adjusted to deliver 20ml/kg at a rate of 35 inhalations/minute.

The right jugular vein was then cannulated with polythene tubing (Portland Plastics, PP30) previously filled with 0.9% w/v saline containing 10 units/ml heparin. The right common carotid artery was

cannulated with polythene tubing (PP30) filled with heparinised saline, and the arterial blood pressure was measured by means of a blood pressure transducer (Bell & Howell, Type 4-327-L221) connected to a Devices M4 or M2 recorder.

Electrical stimulation of the sympathetic outflow of the spinal cord was performed as described by Gillespie & Muir (1967). The indifferent electrode, a steel hypodermic needle, inserted subcutaneously into the left hind limb, was connected to one pole of a square-wave stimulator (Scientific & Research Instruments Ltd.). The other pole was connected to the shaft of the pithing rod. In these experiments, the preparation was injected intravenously with atropine sulphate (1mg/kg) and d-tubocurarine hydrochloride (3mg/kg). The sympathetic outflow was stimulated with supramaximal strength pulses (80v) of 1msec. duration at frequencies ranging from 0.25 to 1.0 Hz for periods of 40 sec. repeated at intervals of 20 or 30 min. Intravenous injection volumes were 0.5ml/kg followed by a "flush" of 1.0ml/kg of 0.9% w/v saline.

* Fluor: 5.0g of PPO and 0.3g of POPOP/litre of toluene
PPO: 2,5 - diphenyloxazole
POPOP: p-bis (2- (5-phenyloxazolyl)) benzene

DRUGS USED

O-Acetylcholine Bromide	-	British Drug Houses
L-Adrenaline Acid Tartrate	-	Koch Light
L-Arterenol Bitartrate	-	Sigma
Ascorbic Acid	-	BDH
Atropine Sulphate	-	BDH
Bethanidine Sulphate	-	Burroughs Wellcome
BOL-148 (Bromo-Lysergic Acid Hydrogen Tartrate)	-	Sandoz
Cocaine Hydrochloride	-	BDH
Desmethyl Imipramine	-	CIBA
Edecrin (Ethacrynic Acid as Sodium Ethacrynate)	-	Merck Sharp & Dohme
EDTA (Ethylene Diamine Tetra Acetic Acid)	-	BDH
EGTA (Ethylene Guanidine Tetraacetic Acid)	-	BDH
Guanethidine Sulphate	-	CIBA
Histamine Acid Phosphate	-	BDH
5-Hydroxytrydtamine Creatinine Sulphate Complex	-	Sigma
Hypertensin (Angiotensin II AMIDE)	-	CIBA
D,L - Isoprenaline Sulphate	-	BDH
Lasix (Frusemide)	-	Hoechst Pharmaceuticals
Methysergide Hydrogen- Maleate	-	Sandoz
D,L - ^{14}C - Noradrenaline Bitartrate	-	Radiochemical Centre, Amersham
Nembutal (Pento Barbiton Sodium	-	Abbotts Laboratories
Pargyline Hydrochloride	-	Abbotts Laboratories
Phentolamine Methane Sulphonate	-	CIBA
Reserpine Injection (Reserpine Hydrochloride)		Halewood Chemical Co
Sarcalasin (Sarc^{1}-Ala^{8}-Angiotensin II,P-113)		Norwich Pharmacal Co
SKF-525-A (Diethyl Amino Ethyl Diphenyl Propyl Acetate)	-	Smith Kline French
Strophanthin G (Ouabain Octahydrate)	-	Sigma
Tubarine (Tubocurarine Chloride)	-	Burroughs Wellcome
Tyramine Hydrochloride	-	Sigma

N.B. DDC is Diethyl Dithiocarbamate.

All drugs are expressed as base throughout the thesis.

CHAPTER I

THE INTERACTION OF ANGIOTENSIN II WITH ENDOGENOUS AND EXOGENOUS NORADRENALINE IN ISOLATED PERFUSED VASCULAR BEDS

INTRODUCTION

Various roles have been ascribed to angiotensin II in the aetiology and maintenance of hypertension. One of the main difficulties is a correlation of plasma levels of angiotensin II to the hypertensive state. In clinical and experimental hypertension, both increased (Page, 1940; Kahn, Skeggs, Shumway & Wisenbaugh, 1952; Judson & Helmer, 1965) and normal (Dexter & Haynes, 1942; Taquini & Fasciolo, 1946; Pickens, **et al**, 1965) levels of circulating pressor substance have been measured.

Infusions of angiotensin II into whole animals induce a transient hypertension at high concentrations (Gross, Bock & Turain, 1961; Brown, Chapius & Robertson, 1963; Day, McCubbin & Page, 1965) whereas continuous infusions of smaller doses leads to a well sustained hypertension (Page & Olmsted, 1961; Brown, Chapius & Robertson, 1963; Dickinson & Lawrence, 1963; McCubbin, de Moura, Page & Olmsted, 1965; Yu & Dickinson, 1965; Dickinson & Yu, 1967). This sustained hypertension is dependent on the presence of an intact peripheral sympathetic nervous system (McCubbin et al, 1965; Dickinson & Yu, 1967). Interactions of angiotensin II with the sympathetic

nervous system are well documented (for review, see Lowe & Scroop, 1970; Starke, 1972a), both in vivo and in vitro.

Isolated rabbit vascular beds were used in the following series of experiments, to examine, in vitro, a possible interaction of infusions of low concentrations of angiotensin II with other vascular agonists.

RESULTS

Isolated ear artery

Stimulation parameters The isolated ear artery responded with constrictions to electrical stimulation of the sympathetic nerves in the range of 1 to 20 Hz, exogenous noradrenaline as acute intra-arterial injections from 10^{-8} to 5×10^{-7} g and angiotensin II as acute intra-arterial injections from 5×10^{-7} to 10^{-3} g. Noradrenaline is more potent than angiotensin II in causing vaso-constriction, both on a molar and a weight/weight basis, a result in marked contrast to the whole animal, where angiotensin II is the more potent pressor compound (McCubbin & Page, 1963)

Effect of infusions of angiotensin II In each preparation, after obtaining steady control responses to spasmogens, infusions of angiotensin II were commenced, and continued for 20 to 30 min. During this period at least four responses to each agonist were recorded. Dose cycles of agonists were not altered at the start of the angiotensin II infusion, to avoid an effect of a time-lag in the dose cycle being confused with any actual interaction between the infused angiotensin II and the agonist.

Infusions of angiotensin II at 10^{-10} g/ml had no effect on the basal perfusion pressure of the ear artery, and had a variable effect on the responses of the artery to nerve stimulation (3Hz) and acute

injections of noradrenaline (50ng). In three out of five experiments, angiotensin II had no effect on the responses to either nerve stimulation or noradrenaline, in one experiment there was a 20% potentiation of the response to nerve stimulation, with no effect on noradrenaline responses, and in the remaining experiment responses to both nerve stimulation and noradrenaline were depressed during the infusion and did not recover subsequently.

Infusions of angiotensin II at 6.25×10^{-9} g/ml did not effect the basal perfusion pressure of the ear artery and in each of the six preparations tested had no effect on the responses to noradrenaline. In three preparations the response to nerve stimulation was potentiated by an average of 20%, the percentage potentiation decreasing as the infusion of angiotensin II was continued; responses to nerve stimulation returned to control values at the end of the infusion. One of these experiments is illustrated in Fig.3. In the other three experiments, infusions of angiotensin II had no significant effect on the response to nerve stimulation.

Infusions of angiotensin II at 2.5×10^{-6} g/ml. did not affect the basal perfusion pressure of the ear artery, and in five experiments exhibited a variable action on the responses to agonist. In three out of five experiments angiotensin II induced a significant

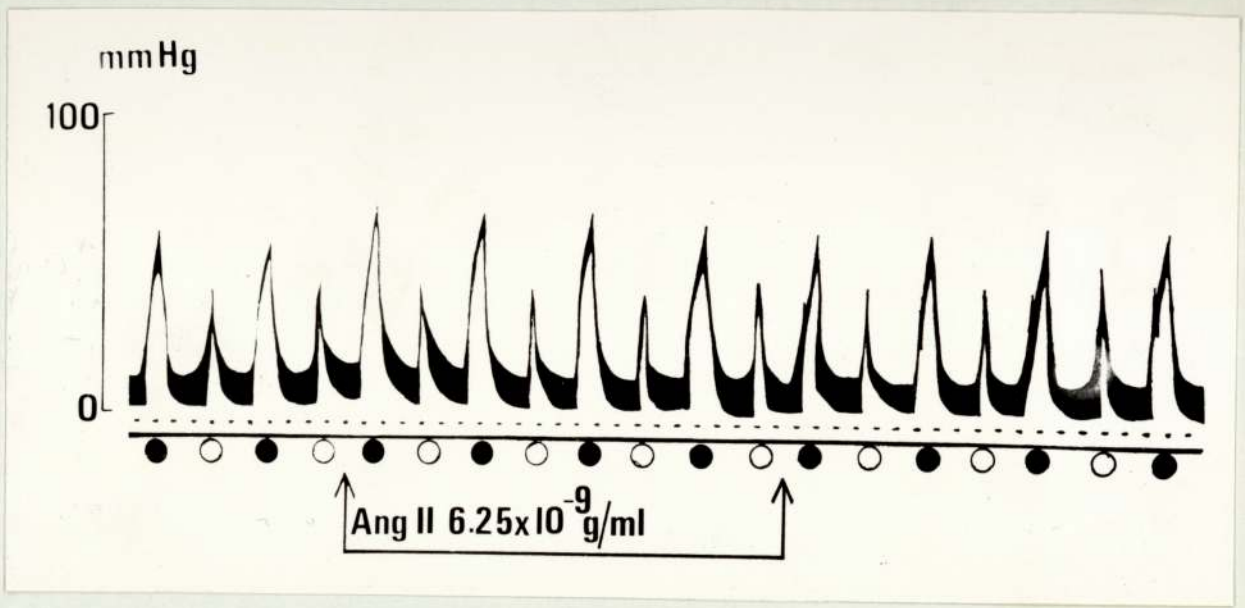

Fig.3 Rabbit isolated ear artery.
Constrictor responses to nerve stimulation (3Hz) - •, and noradrenaline (5×10^{-8} g) - o, angiotensin II (6.25×10^{-9} g/ml) was infused during the period shown.

depression of the responses to both nerve stimulation and noradrenaline; the responses returned to control levels at the end of the infusion. In one experiment there was a slight potentiation (10%) of the response to nerve stimulation at the start of the angiotensin II infusion followed by a depression, again returning to control values on cessation of the infusion, while there was no effect on the response to noradrenaline. In the final experiment, angiotensin II had a potentiating action on the responses to noradrenaline and nerve stimulation, a maximum increase of 50% in each case towards the end of the infusion; on cessation of the infusion responses to both noradrenaline and nerve stimulation returned to control values.

This rather confused picture of the interaction of angiotensin II with noradrenaline and nerve stimulation gave little information as to the true nature of the interaction. It is possible that part of the variability may have been due to a varying degree of access of angiotensin II to the periarterial nerves, which are located in the outer part of the artery (Norberg & Hamberger, 1964). In an attempt to eliminate this variability, arteries were exposed to angiotensin II on their outer coats, either by superfusion, or by cannulating the artery at both ends and incubating in a horizontal organ bath (see methods).

Effects of extra-luminal angiotensin II In each experiment, exposure of the artery to extra-luminal angiotensin II, either in the outer bath or via superfusion, was continued for 20 to 30 min. during which period at least four responses to each agonist were recorded. In each of three experiments, superfusion of the artery with angiotensin II (10^{-10} g/ml) decreased the response to nerve stimulation (3Hz) while having no effect on the response to noradrenaline. On cessation of the infusion, the responses returned to control levels. Similar observations were made in three experiments with double-cannulated arteries exposed to extra-luminal angiotensin II at a concentration of 10^{-10} g/ml in the outer bath. The responses to nerve stimulation were depressed on average 25% and this effect was reversible on removing angiotensin II. A typical experiment is illustrated in Fig. 4(a). Exposure of the artery to extra-luminal angiotensin II, either as a 10^{-10} g/ml perfusion or 10^{-10} g/ml in the bath, had no effect on the basal perfusion pressure of the isolated artery.

Superfusion of the artery with angiotensin II (6.25×10^{-9} g/ml) had a variable effect in four experiments; it induced a small (10%) potentiation of the response to nerve stimulation in two experiments, and had no effect in two further experiments. Responses to noradrenaline were unaffected in each experiment. Addition of angiotensin II (5×10^{-9} g/ml) to the fluid bathing the adventitial surface of the double-cannulated

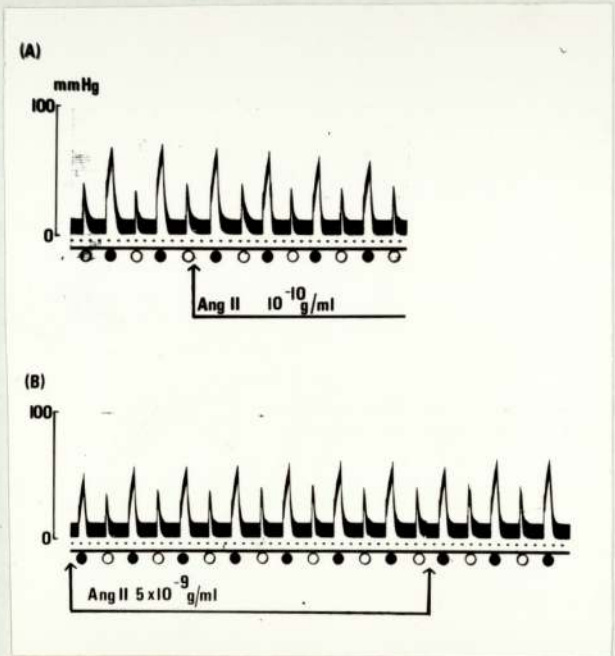

Fig.4 Rabbit isolated ear artery.
Constrictor responses to noradrenaline (5×10^{-8} g) - o, and nerve stimulation (3Hz) - •; during contact of the outer surface of the tissue with angiotensin II (10^{-10} g/ml) (A) and with angiotensin II (5×10^{-9} g/ml) (B)

ear artery had, in four experiments, a depressant action on the responses to nerve stimulation and no effect on the responses to noradrenaline. From the experiment illustrated in Fig. 4(b), it can be seen that the depression induced by this concentration of angiotensin II is less than that induced by lower concentrations, as there appears to be a reversal of the depression during contact with angiotensin II at 5×10^{-9} g/ml. Again, exposure of the outer surface of the artery to angiotensin II at the above concentration had no effect on the basal perfusion pressure.

In each of three experiments, superfusion of the artery with angiotensin II at a concentration of 10^{-6} g/ml had a depressant effect on responses to nerve stimulation and noradrenaline, inducing total blockade after three successive stimulations. This blockade was reversible on cessation of the infusion. Similar observations were made in three experiments when the artery was bathed in a solution of angiotensin II at 10^{-6} g/ml. Again exposure of the outer surface of the artery to angiotensin II at the above concentrations had no effect on the basal perfusion pressure.

Isolated Whole Ear

Stimulation Parameters Responses to nerve stimulation and noradrenaline were similar in whole ear and in isolated ear artery preparations (compare Figs. 4 and 6) However, the whole ear exhibited a greater sensitivity to the direct constrictor response to angiotensin II,

in agreement with the original report of de La Lande & Rand (1965). Whereas the isolated ear artery showed no response to infusions of angiotensin II at a concentration of 2.5×10^{-6} g/ml, seven out of thirteen whole ears gave a significant rise in basal perfusion pressure (25-30mm Hg) on infusion of 6.25×10^{-9} g/ml of angiotensin II.

Effect of phentolamine on constrictor responses to angiotensin II Acute intravenous injections of angiotensin II (2.5×10^{-7}g) gave repeatable constrictor responses in each of five preparations. On addition of phentolamine, in a concentration of 2×10^{-6} g/ml to the perfusing fluid, responses to periarterial nerve stimulation (3Hz) and acute intravenous noradrenaline 10^{-7} g were abolished; however, responses to angiotensin II were potentiated by an average of 50% in the presence of phentolamine. A typical experiment is illustrated in Fig.5. This suggests a lack of direct involvement of endogenously released noradrenaline in the acute constrictor response to angiotensin II via the α-receptor.

Effect of infusions of angiotensin II Infusions of low concentrations of angiotensin II (12.5×10^{-12} g/ml) had no effect on the basal perfusion pressure of the whole ear, and in each of four preparations had no effect on the responses induced by nerve stimulation (3Hz), noradrenaline (10^{-7} g) or 5-HT (10^{-7} g).

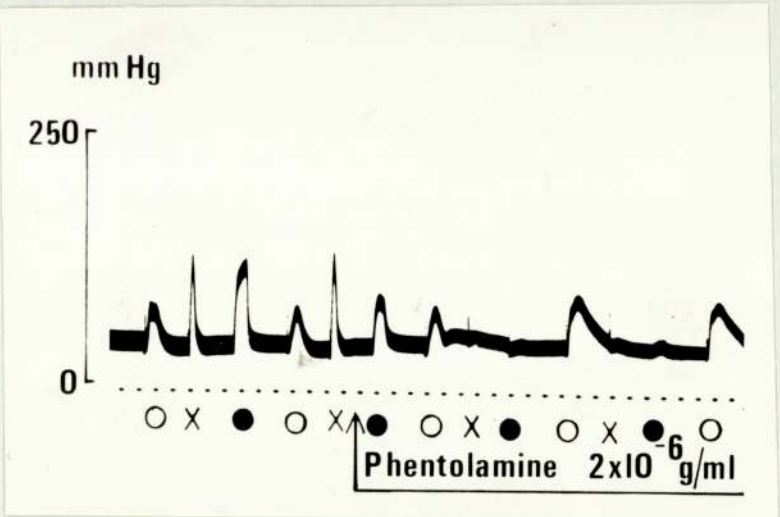

Fig.5 Rabbit perfused ear.
Vasoconstrictor responses to angiotensin II (2.5 x 10^{-7} g) -O, nerve stimulation (3Hz) - • and noradrenaline (10^{-7} g) -X, and the effect of an infusion of phentolamine (2 x 10^{-6} g/ml).

Infusions of higher concentrations of angiotensin II, 6.25×10^{-9} g/ml, for periods of 15 to 30 min. were observed to have one of two effects, both of which were reversible on ending the infusion :-

(a) In six preparations, the infusion of angiotensin II itself had no effect on the basal perfusion pressure; during the infusion responses to nerve stimulation were enhanced, initially, by an average of 85%. The degree of enhancement of the responses to nerve stimulation dedreased during the course of the infusion, and the responses decreased to control levels on cessation of the infusion. Responses to noradrenaline were reduced to an average of 30% of their control size during the infusion and remained at this level after cessation of the infusion. A typical experiment is illustrated in Fig.6 where the effect of a second infusion is also seen. It was found in all six preparations that the potentiating action of a second infusion of angiotensin II was decreased in degree and duration in comparison with that of the first infusion. This may be due to tachyphylaxis to the action of angiotensin II.

(b) In seven preparations, infusion of angiotensin II at a concentration of 6.25×10^{-9} g/ml induced a significant increase in basal perfusion pressure (25-30mm Hg), as can be seen in Fig.7. During the infusion of angiotensin II responses to nerve stimulation were almost totally abolished, whereas the height of the response to noradrenaline was unaffected; it was noted,

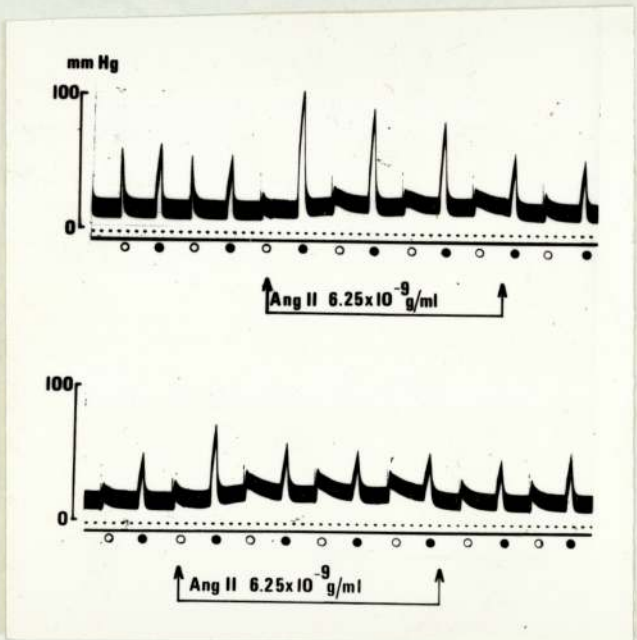

Fig.6 Rabbit perfused ear.

Vasoconstrictor responses to nerve stimulation (3Hz) - •, and noradrenaline (5×10^{-8} g) - o, and the effect of an infusion of angiotensin II (6.25×10^{-9} g/ml). A second infusion of angiotensin had a less marked effect.

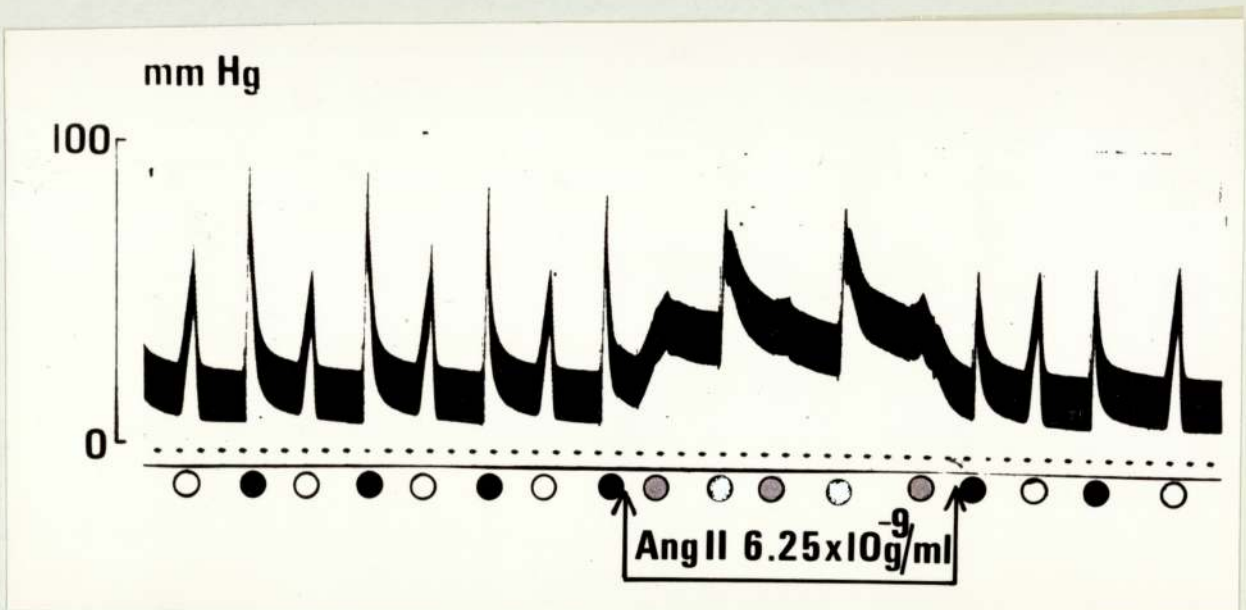

Fig.7 Rabbit perfused ear.

Constrictor responses to nerve stimulation (3Hz) - •, and noradrenaline (5×10^{-8} g) - o, and the effect of an infusion of angiotensin II (6.25×10^{-9} g/ml).

however, that in each preparation the response to noradrenaline was prolonged during the infusion. On cessation of the infusion, both the blockade of the response to nerve stimulation and the prolongation of the response to noradrenaline were reversed.

Isolated mesenteric vascular bed

Stimulation parameters This vascular bed was less sensitive than either the isolated ear artery or the whole ear to both peri-arterial nerve stimulation and acute injections of noradrenaline. The comparatively poor response to noradrenaline was the most significant difference. The sensitivity to the direct constrictor action of angiotensin II was closer to that of the isolated ear artery than the whole ear.

Effect of infusions of angiotensin II In four experiments, the acute intra-arterial injection of 10^{-9} g of angiotensin II, while not itself inducing a constriction, induced a potentiation of the response to nerve stimulation. The typical experiment illustrated in Fig.8 shows this potentiation, which was, on average 100%, i.e. the response to nerve stimulation was doubled in the presence of angiotensin II.

Infusions of angiotensin II in the concentration range 6.67×10^{-10} to 6.7×10^{-8} g/ml induced an increase in the response to nerve stimulation, while having no effect on the responses to noradrenaline. Fig.9 illustrates the effect of an infusion of

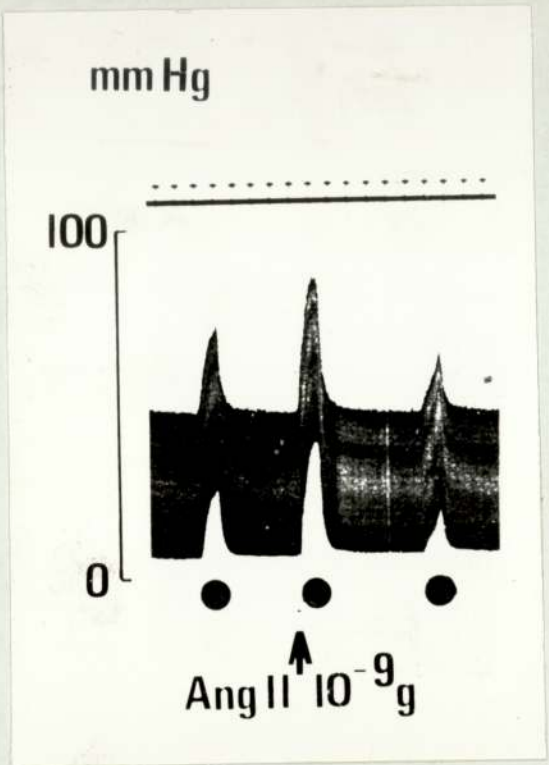

Fig. 8 Rabbit isolated perfused mesenteric bed. Constrictor responses to nerve stimulation (20Hz) - •, and the potentiating action of an acute injection of angiotensin II (10^{-9} g).

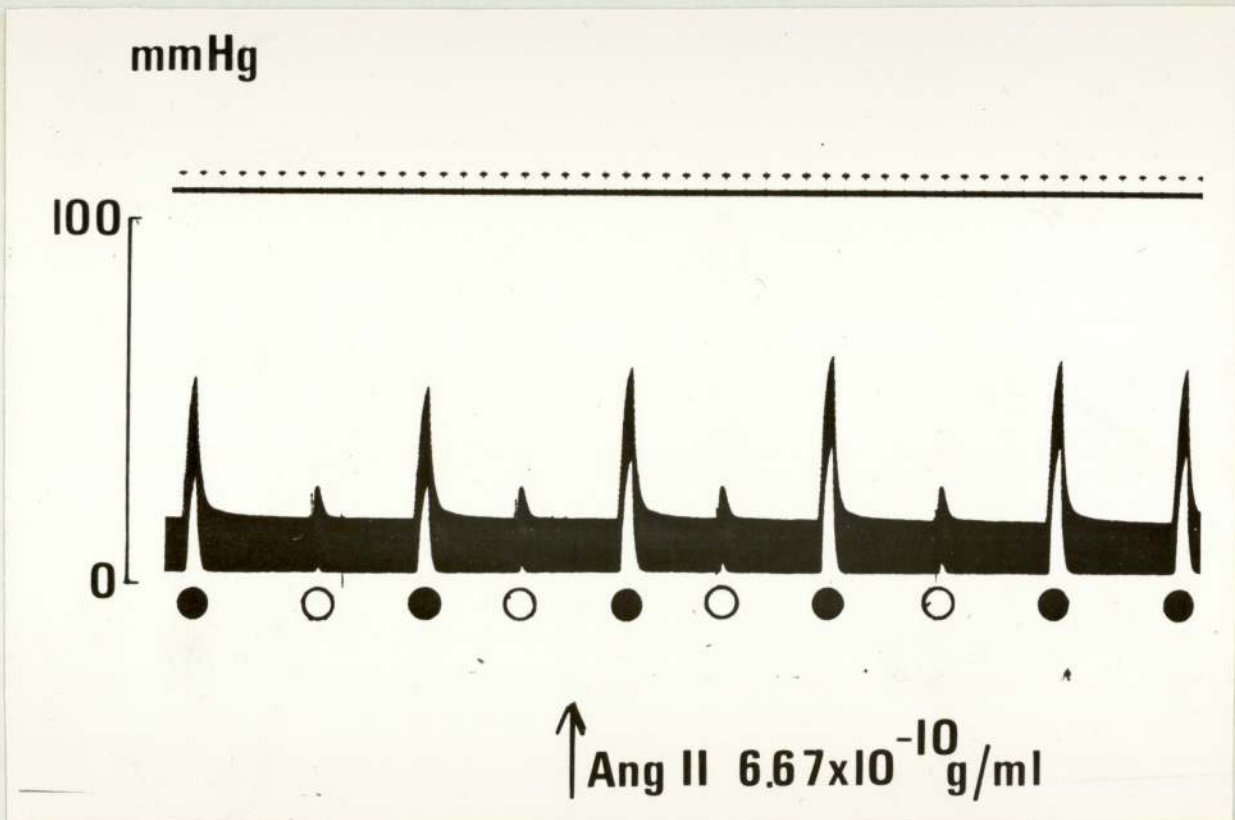

Fig. 9 Rabbit isolated perfused mesenteric bed. Vasoconstrictor responses to nerve stimulation (20Hz) - •, and noradrenaline (6.0 x 10^{-7} g) - o, and the effect of an infusion of angiotensin II (6.67 x 10^{-10} g/ml).

angiotensin II at 6.67×10^{-10} g/ml on responses of the mesenteric bed to nerve stimulation (20Hz) and noradrenaline (6.0×10^{-7} g). The average degree of potentiation in four experiments was 30%. Using the same preparation, an infusion of 6.7×10^{-8} g/ml of angiotensin II had a much greater potentiating action on nerve stimulation, as shown in Fig.10(a). This was true of the other three preparations. The results from the trace in Fig.10(a) are expressed graphically in Fig.10(b), where it can be seen that angiotensin II had a greater percentage potentiating action on the lower frequency of stimulation. On cessation of the angiotensin II infusion, responses to nerve stimulation returned to control values.

A similar degree of potentiation of the responses to nerve stimulation was seen when the mesenteric bed was perfused with angiotensin II at concentrations as low as 4.0×10^{-9} g/ml. Fig.11 shows the results from one of four experiments in which this rate of infusion was repeated on the same preparation. In each experiment, it was found that the second infusion had little, if any, potentiating activity on the response to nerve stimulation whereas the first infusion had a significant action. These findings are similar to those for the whole ear, illustrated in Fig.6.

<u>The effect of nerve stimulation on the direct constrictor response to angiotensin II</u> Acute intra-arterial injections of angiotensin II (2×10^{-6} g) did not constrict in each of five preparations. However,

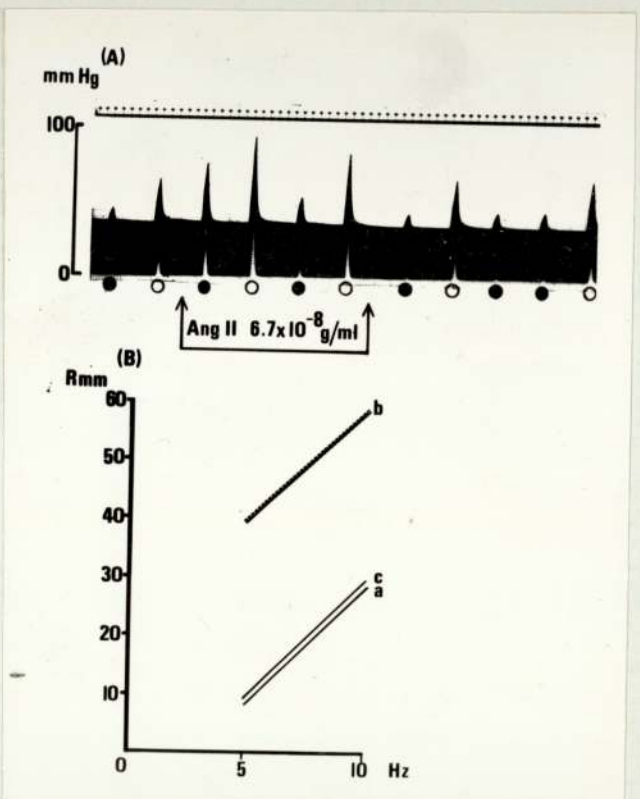

Fig. 10 Rabbit isolated perfused mesenteric bed. Potentiation of constrictor responses to nerve stimulation at 5Hz and 10Hz by an infusion of angiotensin II (6.7×10^{-8} g/ml). Responses in (A) are expressed graphically in (B).

(a) before, (b) during, and (c) after the infusion of angiotensin II.

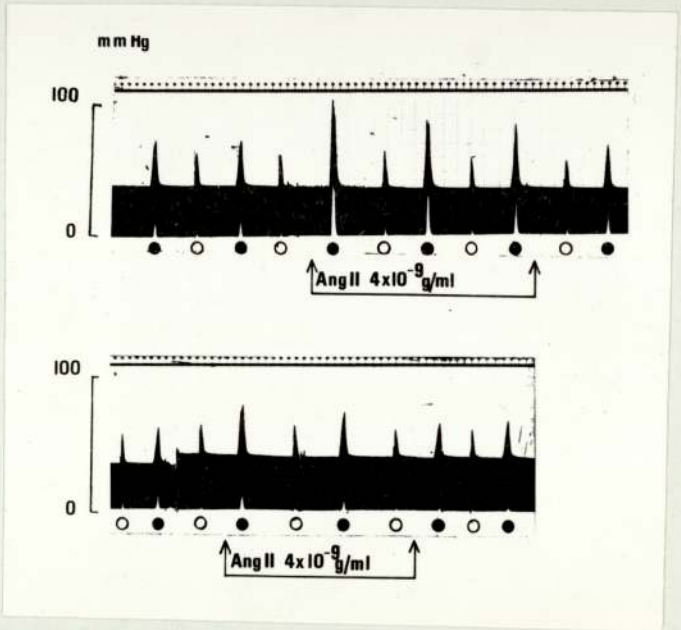

Fig. 11 Rabbit isolated perfused mesenteric bed. Constrictor responses to nerve stimulation (10Hz) - •, and noradrenaline (10^{-6} g) - o, and the effect of an infusion of angiotensin II (4×10^{-9} g/ml). A second infusion of angiotensin II (4×10^{-9} g/ml) has a much smaller potentiating action.

during sympathetic nerve stimulation, the same concentration of angiotensin II did induce a constriction. Fig.12 shows a typical experiment where exposure of the preparation to phentolamine in the perfusion fluid, at a concentration of 5×10^{-6} g/ml, reduced, but did not block, the response to nerve stimulation. However, the previous sub-threshold concentration of angiotensin II does, in the presence of phentolamine, induce a small constriction. The presence of phentolamine blocked the sympathetically-mediated potentiation of the response to angiotensin II.

Discussion

Many investigators have observed that the constrictor potency of angiotensin II varies within different vascular beds. Barer (1961) found that angiotensin II increased the resistance in every canine vascular bed into which it was injected directly. He observed a greater vasoconstrictor activity on the renal and mesenteric beds than on the coronary and pulmonary systems. Using both the dog perfused forelimb and the perfused mesenteric vascular bed, Haddy et al (1962) found that angiotensin II on a weight or molecular basis, was a stronger vasoconstrictor agent than either noradrenaline or 5-HT. The evidence of other investigators is described in the INTRODUCTION.

The general insensitivity of in vitro vascular preparations to the constrictor activity of angiotensin II, in comparison with its potent in vivo constrictor activity, has been observed by several investigators

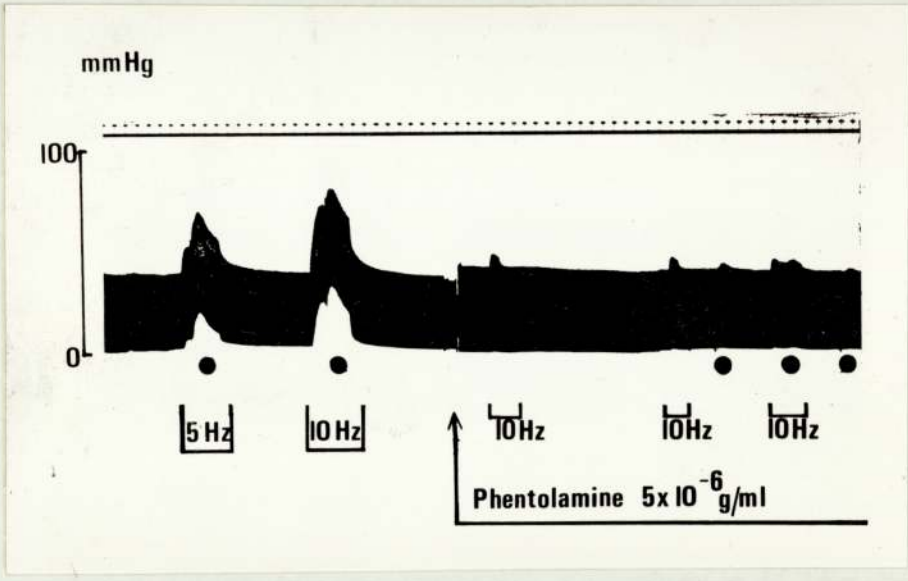

Fig. 12 Rabbit isolated perfused mesenteric bed. Constrictor responses to angiotensin II (2×10^{-6} g) - •, and the potentiating effect of nerve stimulation (5Hz and 10Hz) on these responses. During an infusion of phentolamine (5×10^{-6} g/ml) this potentiating action was blocked.

(de la Lande & Rand, 1965; McGregor, 1965; Bohr & Uchida, 1967; Day & Owen, 1968). Three factors may operate in regard to the ear artery :-

(i) Zimmerman (1962) found that constrictor responses to angiotensin II, but not those to noradrenaline, were reduced by acute sympathectomy of the perfused dog hindlimb. As there is no sympathetic tone in isolated vessels, it is probable that this lack of tone contributes to the lack of sensitivity. The degree of sympathetic tone in blood vessels has been shown to regulate the sensitivity of the tissue to angiotensin II as Day & Owen (1968) found, using the isolated ear artery, that during periarterial nerve stimulation responses to angiotensin II were enhanced, the enhancement being prevented by reserpinisation or exposure of the tissue to guanethidine or phentolamine. Similar observations were reported on the isolated mesenteric bed in the results section of this chapter.

(ii) Absence of blood-borne factors; Ng, Teh & Whelan (1971) have shown that perfusion of the rabbit ear artery with diluted rabbit serum increases the responsiveness to angiotensin II.

(iii) From the results section, it can be seen that the whole perfused ear is more sensitive to the constrictor action of angiotensin II than the isolated artery, thus the absence of arterioles may contribute to this insensitivity, as suggested originally by de la Lande & Rand (1965).

Infusion of the isolated ear artery with angiotensin II had such a variable effect on the responses to spasmogens that no conclusions can be drawn from the results. However, exposure of the extra-luminal surface of the artery to angiotensin II had quite definite effects: a selective depression of responses to nerve stimulation at lower concentrations, and a non-specific depression in the higher range. Day & Owen (1968) previously reported a non-specific depressant action of angiotensin II, either acutely or as an infusion, in the isolated ear artery. Thus, the effect of low concentrations of angiotensin II appears to be a specific anti-nerve effect, but this effect is not of sufficient magnitude to account for the almost total blockade at higher concentrations.

In the whole ear, infusions of angiotensin II at sub-constrictor levels potentiated responses to nerve stimulation and reduced those to noradrenaline, whereas infusions of angiotensin II causing a constrictor response blocked the effects of nerve stimulation while prolonging the action of noradrenaline. In the latter case, angiotensin II appears to be acting like an adrenergic neurone blocking drug, whereas in the former case, the mode of action is very difficult to understand. Many mechanisms have been proposed to explain the interaction of angiotensin II with the sympathetic nervous system; as outlined in the INTRODUCTION. The mechanism suggested by

Palaic & Khairallah (1967a) of a neuronal-noradrenaline uptake-blocking action of angiotensin II would appear to explain one set of observations; possibly in the set of experiments where angiotensin II induced a depression of the response to noradrenaline, it was causing release of excessive amounts of endogenous noradrenaline during nerve stimulation, and hence inducing a tachyphylaxis at the level of the α-receptor.

The potentiating action of angiotensin II on responses to nerve stimulation was poorly repeatable in the same tissue; if angiotensin II induces an increase in the response to nerve stimulation by causing release of more noradrenaline per nerve impulse, as has been suggested by Day & Owen (1968) and Hughes & Roth (1969), it may be proposed that angiotensin II is causing a depletion of transmitter, hence the attenuation of its potentiating action. However, this is unlikely as the size of the control responses to nerve stimulation remains constant throughout the experiment (see Fig.6). Thus it appears that there is a development of tachyphylaxis to the potentiating action of angiotensin II.

Angiotensin II exhibited a powerful potentiating action on the mesenteric bed. At all concentrations tested, it induced a potentiation of the responses to nerve stimulation, while having no effect on the responses to noradrenaline. As for the whole ear, there was development of tachyphylaxis to the potentiating action of angiotensin II.

The direct constrictor action of angiotensin II was potentiated during sympathetic nerve stimulation in the mesenteric bed; this potentiation was blocked in the presence of the α-adrenergic receptor blocking drug phentolamine. Similar observations were made on the isolated ear artery by Day & Owen (1968). However, the direct constrictor action of angiotensin II was potentiated in the presence of phentolamine itself, both in the isolated whole ear and the isolated mesenteric bed. Pals & Fulton (1968) suggested a synergism between noradrenaline and angiotensin II at the level of the vascular α-receptor, but it is difficult to correlate this with the action of phentolamine. One of several hypotheses proposed by Panisset & Bourdois (1968) was that angiotensin II is taken up either onto or into the sympathetic nerve ending, hence limiting its vasoconstrictor activity; it would then be released during sympathetic nerve stimulation, so that an apparent potentiation of the response would occur during sympathetic nerve stimulation. The action of phentolamine could then be explained in two ways :-

(1) α receptors, or part of the α receptor, on smooth muscle act as "spare" receptors for angiotensin II, not contributing to the musculotropic activity; phentolamine would prevent this binding to spare receptors.

(2) Pre-synaptic α receptors (Starke, 1972b) are involved in the binding of angiotensin II to the nerve membrane, and phentolamine would prevent this pre-synaptic binding.

Both suggestions are extremely hypothetical, and necessitate angiotensin II binding studies.

CHAPTER II

THE INTERACTION OF ANGIOTENSIN II WITH ENDOGENOUS AND EXOGENOUS NORADRENALINE IN THE RABBIT ISOLATED AORTA

INTRODUCTION

The perfused artery and arterial bed preparations described in the first chapter displayed a variability in response to the effect of angiotensin II in potentiating responses to endogenous noradrenaline and a relative insensitivity to the direct constrictor response to angiotensin II. In the present chapter, the experiments described were performed on the rabbit isolated spirally-cut thoracic aorta; this tissue is highly sensitive to the direct constrictor action of angiotensin II (see Chap.VI) and constant, repeatable responses to most vascular spasmogens may be obtained.

Elchlepp & Furchgott (1955) found an essentially circular orientation of smooth muscle in the rabbit thoracic aorta, and practically no longitudinally orientated smooth muscle. The circular muscle constitutes almost half of the material of the arterial wall. This orientation is important in two respects:-

(i) When the artery is cut spirally, it provides a sheet of longitudinally orientated smooth muscle fibres.

(ii) The direction of cutting of the spiral does not alter the responses, as opposed to the case with ox mesenteric arteries reported by Hausler (1933).

As a preparation for use in the analysis of action of vasoactive drugs, the spirally cut isolated thoracic aorta of the rabbit has been in use since the early 1950's. Furchgott & Bhadrakom (1953) made a detailed study of the responses of this tissue to vasoconstrictor and vasodilator drugs. They found a qualitative similarity to other isolated blood vessel preparations: histamine, acetylcholine noradrenaline, adrenaline, 5-HT and isoprenaline were vasoconstrictor, and sodium nitrite and isoprenaline caused a relaxation when the tone of the tissue had been raised by a spasmogen.

Effective methods of electrically stimulating the periarterial nerves of the aorta without also directly stimulating smooth muscle cells have been suggested, e.g. Yates & Gillis (1963) but Paterson (1965) produced the definitive method. He clearly demonstrated that responses to electrical stimulation using the parameters he defined (See METHODS) were totally due to stimulation of sympathetic nerves in the arterial wall.

The experiments described in this section were performed to elucidate a possible role of angiotensin II as a vascular sensitising agent in both physiological and pathological processes.

RESULTS

Effect of subthreshold concentrations of angiotensin II on noradrenaline-induced contractions of aortic strips

Noradrenaline (10^{-9} to 10^{-6} g/ml) caused dose-related contractions of isolated aortic strips. It was found that a suitable submaximal response could regularly be obtained at a concentration of 10^{-8} g/ml, which gave a contraction of 2-3cm height with the maximal response being reached after 2 to 4 min. These data are similar to those reported by Furchgott & Bhadrakom (1953) for this preparation. In the presence of angiotensin II at concentrations (5.0×10^{-11} to 10^{-9} g/ml) too low themselves to cause contractions of the tissues, the responses to noradrenaline were increased. A typical experiment is illustrated in Fig.13, where contractions induced by noradrenaline (10^{-8} g/ml) were approximately doubled in the presence of angiotensin II (10^{-9} g/ml). After the removal of the angiotensin II from the bath, noradrenaline responses gradually returned to control levels.

When tissues had been exposed to angiotensin II, and responses to noradrenaline had returned to control levels after washout of the angiotensin II, then a further contact of the tissue with angiotensin II induced a similar degree of potentiation of the responses to noradrenaline as when the tissue had first been exposed to angiotensin II. The experiment shown in Fig.14 illustrates this repeatability of the effect.

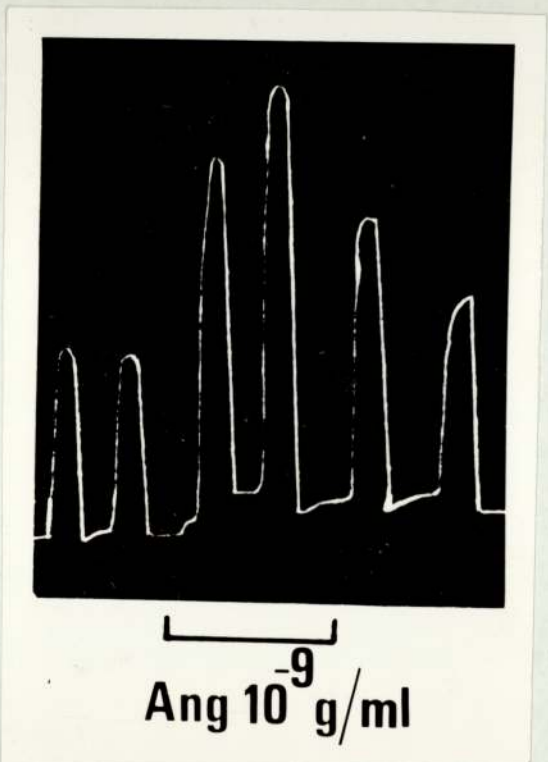

Fig. 13 Rabbit isolated aortic strip.
Responses to noradrenaline (10^{-8} g/ml), during the period shown, angiotensin II (10^{-9} g/ml) was in contact with the preparation.

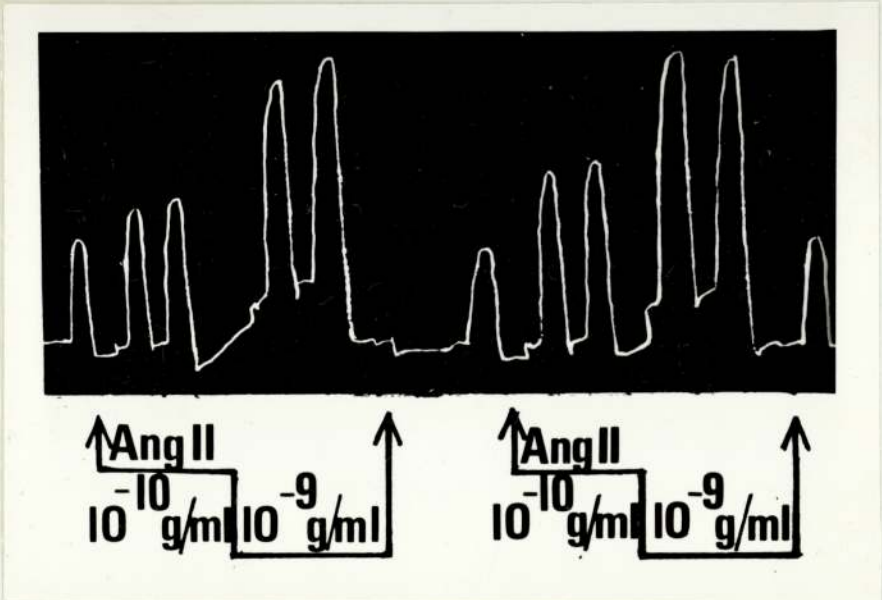

Fig. 14 Rabbit isolated aortic strip.
Potentiation of responses to noradrenaline (10^{-8} g/ml) in the presence of angiotensin II at two concentrations 10^{-10} g/ml and 10^{-9} g/ml. The repeatability of this potentiation is demonstrated.

Effect of angiotensin II on speed and duration of noradrenaline-induced contractions

On addition of noradrenaline (10^{-8} g/ml) to the medium bathing an aortic strip, there was usually a 10 to 20 sec. delay period before any contraction was recorded; during contact of the tissue with angiotensin II (10^{-9} g/ml) there was a shortening of this delay period -- it was, on average, reduced to 50% of the control value. The actual speed of the noradrenaline-induced contraction was also increased, especially in the early stages of the contraction. Fig.15 illustrates a typical experiment where responses to noradrenaline (10^{-8} g/ml) were recorded only in the initial stages of contraction; control responses were recorded for 70 sec. whereas responses to noradrenaline in the presence of angiotensin II (10^{-9} g/ml) were recorded for only 60 sec., so that the significant difference in height between responses does reflect a true difference in speed of responses rather than just a difference in the times between addition of noradrenaline and the commencement of the contraction.

Although responses to noradrenaline were increased both in speed and height in the presence of subthreshold concentrations of angiotensin II, there was no significant increase in the duration of the response.

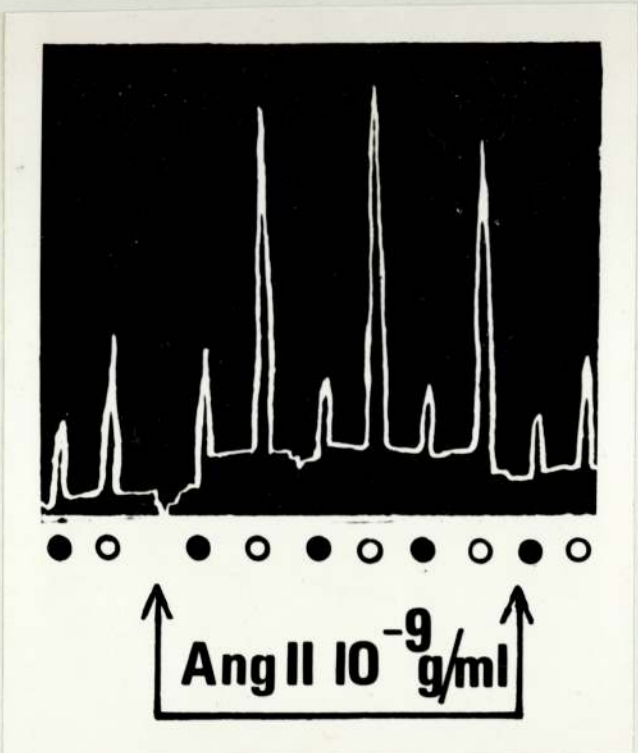

Fig. 15 Rabbit isolated aortic strip.
Responses to noradrenaline (10^{-8} g/ml) and nerve stimulation (10 Hz) recorded for only the first minute of the response only. In the presence of angiotensin II (10^{-9} g/ml) the height of response reached during 1 min. was increased, i.e. the speed of response was increased.

Effect of angiotensin II on dose-response curve to noradrenaline

Angiotensin II (10^{-9} g/ml) induced a greater percentage potentiation of responses to noradrenaline at the lower end of the dose-response curve. Values from a typical experiment are expressed graphically in Fig.16, where responses at the lowest concentration of noradrenaline (10^{-8} g/ml) are potentiated by over 100%, whereas at the higher end of the scale, the percentage potentiation is considerably less.

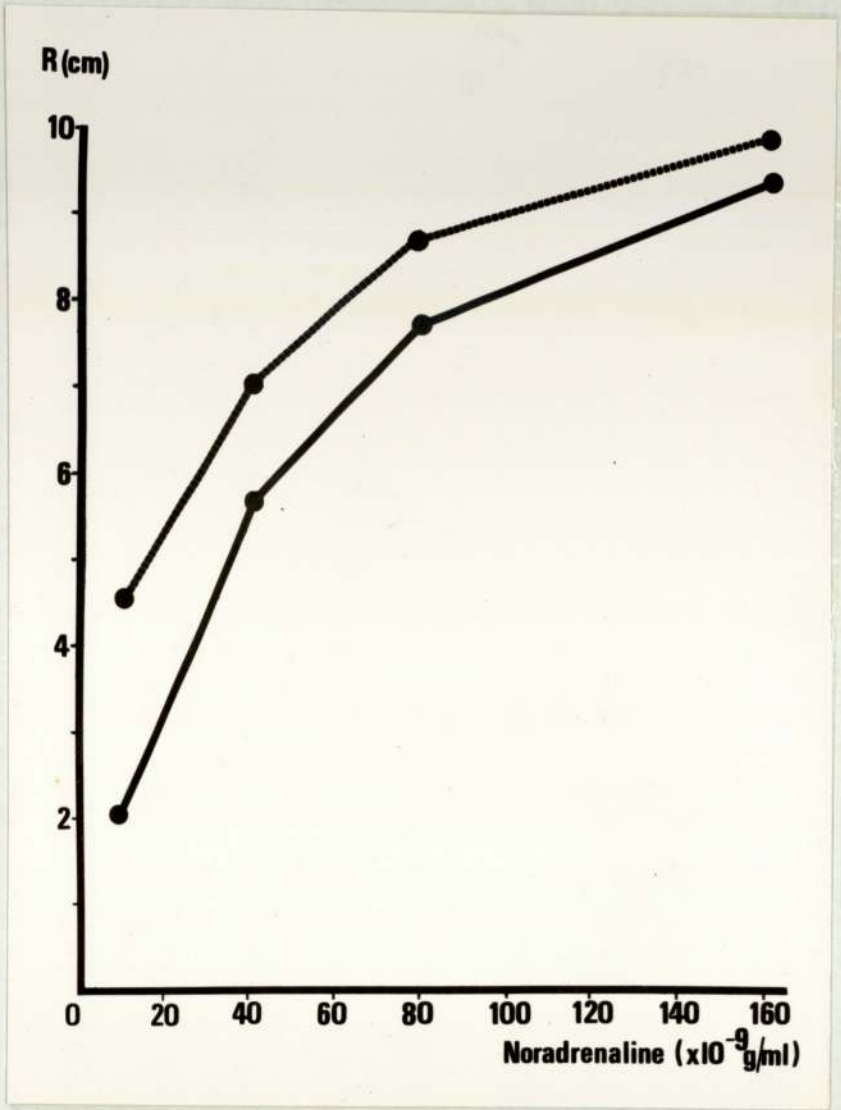

Fig. 16 Rabbit isolated aortic strip.
The potentiating action of angiotensin II (10^{-9} g/ml) on the dose-response curve to noradrenaline, from one aorta. Stippled (upper) curve - in the presence of angiotensin II.

Effect of angiotensin II on responses to procedures releasing endogenous noradrenaline

The artery strip was stimulated by bi-polar electrodes, enclosed in epoxy resin, placed around the artery strip; stimulation parameters were: frequency 10 Hz, pulse width 0.5 msec, voltage 100v applied for 30 sec. The artery usually continued to contract for a further 30 sec. after cessation of stimulation, and this was included in the recording. At the end of each experiment, the artery was exposed to bethanidine (5.0×10^{-4} M) for 30 min. and the nerve stimulation repeated; in all cases, this resulted in a significant reduction (average 85%) in the size of the response, indicating that the response was mediated via the sympathetic nerves supplying the tissue.

Fig.17 shows a typical result of the influence of angiotensin II (10^{-9} g/ml) on the responses to nerve stimulation. The height of the response to nerve stimulation was initially enhanced and then reduced in the presence of angiotensin II. On washout of angiotensin II, the responses to nerve stimulation remained at a lower level than control values. Similar observations were made when the tissue was stimulated with tyramine (10^{-6} g/ml) in place of nerve stimulation. With both tyramine and nerve stimulation, the initial potentiation of responses due to angiotensin II affected only the height of the response - it was not prolonged in duration.

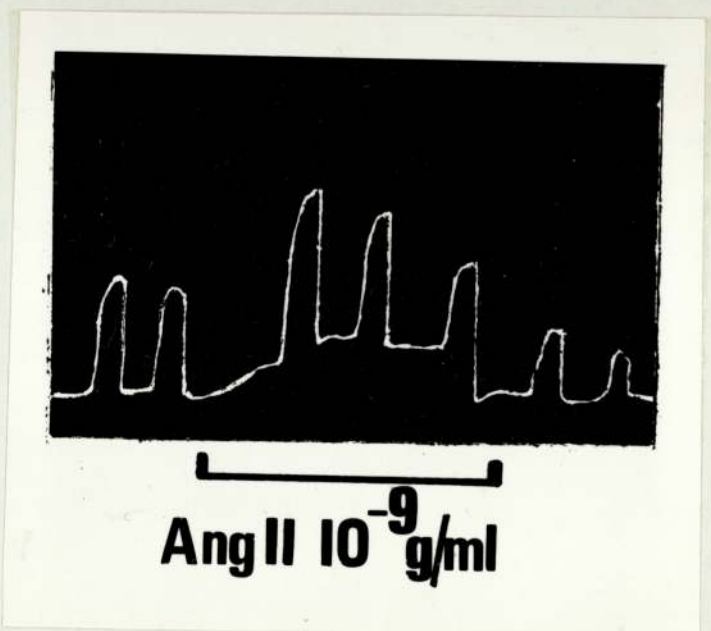

Fig. 17 Rabbit isolated aortic strip.
Responses to nerve stimulation (10Hz), during the period shown, angiotensin II (10^{-9} g/ml) was in contact with the preparation.

During the time course of the experiments, constant responses could be obtained to both nerve stimulation and tyramine, so that the depression of responses seen after washout of angiotensin II appeared to be a consequence of its presence, rather than to tachyphylaxis.

The effect of constrictor concentrations of angiotensin II on responses to noradrenaline

A potentiation of noradrenaline induced contractions was seen with concentrations of angiotensin II as low as 5×10^{-11} g/ml. The maximum potentiation, which was approximately equal to a doubling of the control size, occurred at a concentration around 10^{-9} g/ml. Higher concentrations of angiotensin II, which themselves caused contractions of the aorta, reduced the size of the response to noradrenaline. However, in these experiments after washout of angiotensin II from the bath, it was regularly observed that the noradrenaline responses initially returned to a size greater than that of the control responses. A typical experiment is seen in Fig.18 where there is a significant reduction of the size of the response to noradrenaline (10^{-8} g/ml) at both concentrations of angiotensin II (5.0×10^{-9} and 10^{-8} g/ml). It can be seen in this figure that on washout of the angiotensin II the subsequent response to noradrenaline is greater in size than the control response.

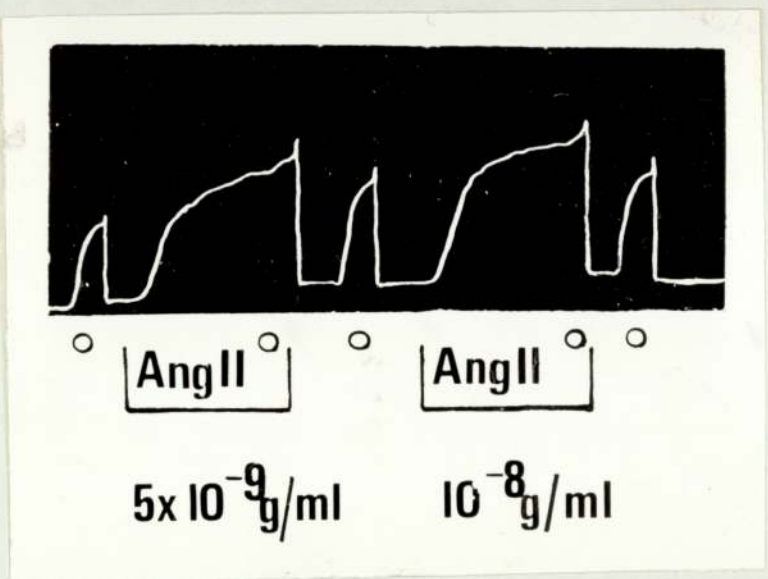

Fig. 18 Rabbit isolated aortic strip.
Responses to noradrenaline (10^{-8} g/ml) - o, and the depressant effect of contractor concentrations of angiotensin II (5×10^{-9} g/ml and 10^{-8} g/ml)

The depression of noradrenaline-induced contractions by constrictor concentrations of angiotensin II was not specific since contractions induced by a variety of agonists were similarly reduced, as can be seen from the table in Fig.19

Effect of P-113 (Sar^1-ala^8-angiotensin II) on angiotensin II-induced potentiation of responses to noradrenaline

P-113 is an angiotensin II analogue, and has been used as an angiotensin II receptor blocking agent in several vascular preparations (Zimmerman, 1973; Rioux, Park & Regoli, 197[illegible]). Exposure of the aorta to P-113 at a concentration of 10^{-8} g/ml induced no alteration in base-line; there was, however, a slight but non-significant increase, in the response to noradrenaline during contact of the tissue with P-113. Fig.20 shows the effect of exposing the tissue to P-113 before and during contact of the tissue with angiotensin II itself. P-113 (10^{-8} g/ml) had no significant effect on the response to noradrenaline (10^{-8} g/ml), but it prevented the angiotensin II-induced potentiation of the responses to noradrenaline. Fig.21 shows the results of a typical experiment, in which addition of P-113 during contact of the tissue with angiotensin II induced a reversal of the enhancing activity on the responses to noradrenaline.

Fig. 19

AGONIST	CONC. N g/ml	% REDUCTION BY ANGIOTENSIN II $5x10^{-9}$ g/ml	±SE	10^{-8} g/ml	±SE	n
Noradrenaline	10^{-8}	86.3	2.0	92.4	3.1	6
Histamine	$1.2x10^{-7}$	69.6	4.7	80.4	6.9	4
Acetylcholine	$4.0x10^{-6}$	70.1	4.4	85.3	1.1	4
Potassium Chloride	$1.11x10^{-3}$	84.3	5.0	94.6	3.7	4
5-HT	$4.0x10^{-8}$	82.3	2.7	84.8	0.6	4

Fig. 19 Effect of contractor concentrations of angiotensin II (5×10^{-9} g/ml and 10^{-8} g/ml) on contractions of rabbit isolated aorta induced by a variety of agonists. Reductions are expressed as percentages of initial responses to each agonist.

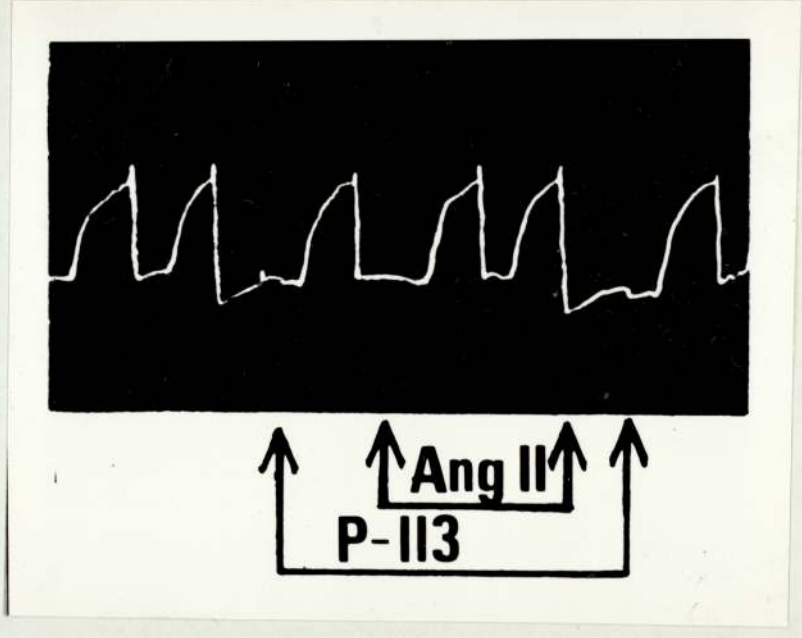

Fig. 20 Rabbit isolated aortic strip.
Responses to noradrenaline (10^{-8} g/ml); the potentiating action of angiotensin II (10^{-9} g/ml) is blocked by p re-exposure of the tissue to P-113 (10^{-8} g/ml).

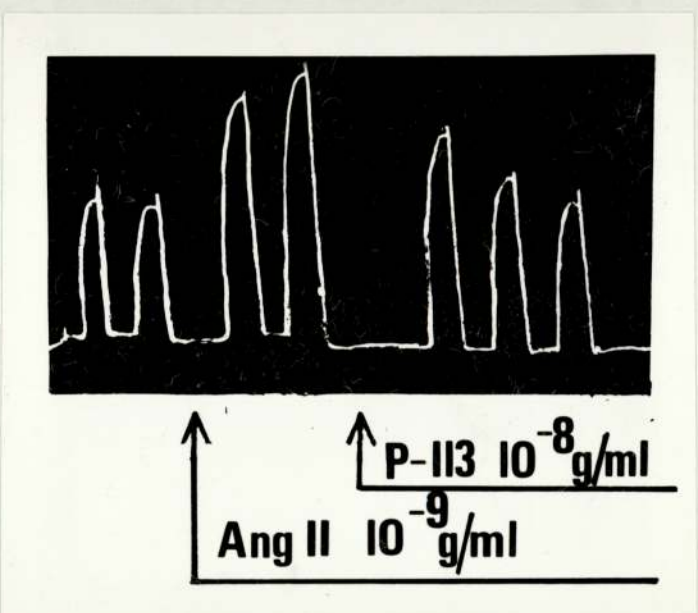

Fig. 21 Rabbit isolated aortic strip. Responses to noradrenaline (10^{-8} g/ml); the potentiating action of angiotensin II (10^{-9} g/ml) is reversed on contact of the tissue with P-113 (10^{-8} g/ml).

Effect of 5-HT on responses to noradrenaline

Subthreshold concentrations of 5-HT (5×10^{-10} to 1.6×10^{-9} g/ml) potentiated the response of the aorta to noradrenaline, a typical experiment being shown in Fig.22. In this example, the responses to noradrenaline were potentiated by 70% of control values in the presence of 5-HT in a concentration of 1.6×10^{-9} g/ml. This concentration was found to be the maximally effective potentiating concentration. The percentage potentiation of responses to noradrenaline by 5-HT was less than by angiotensin II; a maximum of 70% above controls was seen with 5-HT, whereas angiotensin II potentiated responses to noradrenaline to about 100% above control values.

Effect of P-113 on the potentiation of noradrenaline by 5-HT

The presence of P-113, as before, had a slight non-significant potentiating effect on the responses to noradrenaline. The percentage potentiation of noradrenaline induced contractions by 5-HT was not affected in the presence of P-113, one of four experiments being illustrated in Fig.23. The results seen in Fig.22 and 23 are from the same preparation. 5-HT is, therefore, not acting on angiotensin II receptors to induce a potentiation of the responses to noradrenaline.

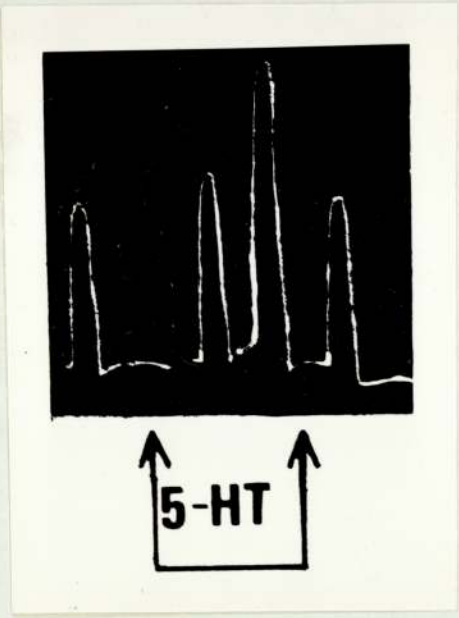

Fig. 22 Rabbit isolated aortic strip.
Responses to noradrenaline (10^{-8} g/ml) are potentiated in the presence of 5-HT (1.6 x 10^{-9} g/ml).

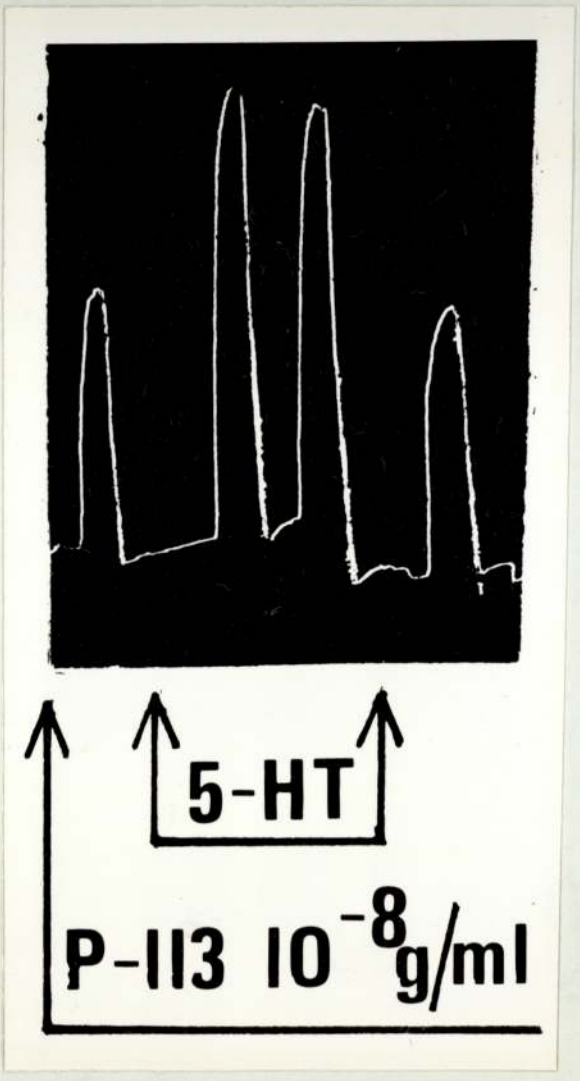

Fig. 23 Rabbit aortic strip.
The potentiation of responses to noradrenaline (10^{-8} g/ml) by 5-HT (1.6×10^{-9} g/ml) is unaffected by contact of the tissue with P-113 (10^{-8} g/ml).

Effect of methysergide and BOL-148 on the potentiation of noradrenaline by 5-HT

Both BOL-148 and methysergide, in a concentration of 10^{-6} g/ml induced an increase in base-line after 30 min. contact with the preparation. Methysergide had no significant effect on the size of the responses to noradrenaline in each of four preparations, but BOL-148 reduced the size of the responses to noradrenaline by approximately 50% in each of four experiments, i.e. at the above concentration, it was acting non-specifically. Both antagonists prevented the enhancement of responses to noradrenaline by sub-threshold concentrations of 5-HT. 5-HT was, therefore, apparently inducing a potentiation of noradrenaline-induced contractions by combination with 5-HT receptors.

DISCUSSION

Interactions of angiotensin II with the sympathetic nervous system and with indirectly acting sympathomimetic amines are well documented, and have been recently reviewed (Lowe & Scroop, 1970; Starke, 1972a). However, interactions between angiotensin II and exogenous noradrenaline have been less consistently reported. In some whole animal preparations, such as anaesthetised dog blood pressure (McCubbin & Page, 1963) and in some isolated organs, such as the rat isolated mesenteric bed (McGregor, 1965) and portal vein (Blair-West & McKenzie . 1971) and cat isolated perfused terminal ileum (Turker, 1973)

angiotensin II did not cause an enhancement of responses to exogenous noradrenaline. However, in the human isolated digital artery (Whelan, Scroop & Walsh, 1969), rat isolated caudal artery (Nicholas, 1970) and perfused hindlimb (Sato & Masuyama, 1971) and the dog's perfused paw (Liao & Zimmerman, 1972) a consistent enhancement occurred. The mechanism of the enhancement of both endogenous and exogenous noradrenaline by angiotensin II is not clear.

In the rabbit isolated aorta, angiotensin II in contact with the tissue in subthreshold concentrations potentiated the responses of the tissue to noradrenaline, tyramine and nerve stimulation. As long as angiotensin II was in contact with the tissue, responses to noradrenaline remained potentiated, whereas responses to tyramine and nerve stimulation decreased. The noradrenaline-potentiating action of angiotensin II was readily repeatable.

Many mechanisms of the interaction between angiotensin II and the sympathetic nervous system have been postulated. Interactions at the level of the brain and ganglia (see INTRODUCTION) can, of course, be discounted in consideration of the isolated aorta. Most theories are concerned with an interaction of angiotensin II with the sympathetic nervous system and/or exogenous noradrenaline at one of two levels; the sympathetic nerve ending, or the vascular smooth muscle cell.

(i) The nerve ending

(a) Palaic & Khairallah, (1967b) proposed that angiotensin II prevents reuptake of noradrenaline into sympathetic nerve endings.

(b) Boadle, Hughes & Roth (1969) found that angiotensin II caused an enhancement of the synthesis of noradrenaline in the sympathetic nerve ending, possibly by removal of end-group inhibition on the enzyme dopamine -β- hydroxylase.

(c) Khairallah, Page & Turker (1966b) found that contractile responses of rabbit isolated aortae to directly acting spasmogens were potentiated by indirectly acting sympathomimetic amines. This potentiation did not occur with aortae taken from reserpinised rabbits. Liebau, Distler & Wolff (1965) reported that angiotensin II had a direct noradrenaline-releasing effect on sympathetic nerve endings in the isolated aortae of several mammals including the pig and rat; thus a further mechanism of interaction may be proposed; angiotensin II releases noradrenaline from sympathetic nerve endings, and this, in turn, sensitises vascular smooth muscle to other spasmogens.

(d) A facilitated release of noradrenaline during sympathetic nerve stimulation has been proposed by Benelli, della Bella & Gandini (1964), Day & Owen (1969) Hughes & Roth (1969, 1971). Palaic & Panisset (1969) observed an inhibition of release of noradrenaline during nerve stimulation in the presence of angiotensin II in the guinea pig isolated vas deferens, possibly

suggesting a mechanism for the inhibitory action of angiotensin II on responses to nerve stimulation in the isolated ear artery of the rabbit, reported by Day & Owen (1968).

(e) Inhibition of noradrenaline-inactivating enzymes, both COMT and MAO.

(ii) Smooth muscle

(a) Pals & Fulton (1968) proposed a post-synaptic site of interaction of angiotensin II with noradrenaline, at the level of the vascular α-receptor.

(b) Thoenen, Hurlimann & Haefely (1965) suggested that angiotensin II had a direct effect on the excitation-contraction coupling process of the cell.

(c) Many reports have linked angiotensin II with a variation in tissue cation levels. As this topic forms the basis for the discussion of Chap.IV and Chap. V, further consideration of these reports is given there.

Of the theories involving the sympathetic nerve ending, as the site of action of angiotensin II, two may be discounted immediately as fully comprehensive theories in the present case, where responses to both endogenous and exogenous noradrenaline are potentiated in the presence of angiotensin II. An enhanced synthesis of noradrenaline in the presence of angiotensin II, and a facilitated release of noradrenaline during sympathetic nerve stimulation would be expected to affect the responses to endogenously released noradrenaline only,

neither of the theories account for the observed potentiation of exogenous noradrenaline by angiotensin II. However, it is feasible that angiotensin II may be acting via different mechanisms in inducing a potentiation of noradrenaline released from endogenous stores and exogenous noradrenaline.

Responses to both tyramine and nerve stimulation were initially potentiated by angiotensin II, but on prolonged contact the responses returned to, or below, control values. However, responses to exogenous noradrenaline were initially potentiated, and remained potentiated throughout contact of the tissue with angiotensin II. This suggests that angiotensin II may be acting at different sites to induce the potentiations, or by different mechanisms, or it may simply reflect a tachyphylaxis to the action of angiotensin II on sympathetic nerve endings.

An inhibition of enzymatic processes involved in the degradation of noradrenaline by angiotensin II is unlikely, as inhibition of MAO would induce a prolonged response to nerve stimulation and tyramine, and inhibition of COMT a prolonged response to noradrenaline (Kalsner, 1968). In all of the preparations studied, a prolongation of the responses to nerve stimulation, tyramine and noradrenaline in the presence of angiotensin II was not observed.

Inhibition of neuronal uptake of noradrenaline appears to be a feasible hypothesis, as does an action on the smooth muscle cell itself. The noradrenaline-potentiating action of angiotensin II is blocked in the presence of P-113, a compound which is known to block the direct constrictor activity of angiotensin II on the rabbit aorta (Rioux, Park & Regoli, 1973) suggesting that angiotensin II is acting via its receptors on the smooth muscle cell, rather than via an action on the sympathetic nerve ending. However, Zimmerman (1973) has recently shown that the potentiating action of sub-contractile concentrations of angiotensin II on the constrictor responses to sympathetic nerve stimulation in the perfused dog hindpaw is also blocked in the presence of P-113. Thus the action of P-113, although demonstrating that angiotensin II is potentiating responses to noradrenaline via an action on angiotensin II-receptors, does not differentiate between the sympathetic nerve ending and the smooth muscle cell as a site for this receptor.

Kiran & Khairallah (1969) found that angiotensin II increased the noradrenaline efflux from sympathetic nerve endings in the rabbit isolated aorta, but only at a much larger dose than is required to produce maximum contractions of the aorta; also Keatinge (1966) has shown that in this dose range, angiotensin II has a depolarising action on isolated blood vessels. Meyer & Baudouin (1971) found that the degree of contraction induced by angiotensin II in vitro on the rabbit aortic

was the same in the presence or absence of the adventitia, i.e. in the presence or absence of the sympathetic nerves. This suggests that neuronal binding of angiotensin II is not significant in the isolated aorta, at the concentrations of angiotensin II used in the present experiments; thus the blocking action of P-113 now suggests that angiotensin II is acting on angiotensin II receptors either on or in the smooth muscle cell to induce a potentiation of exogenous noradrenaline. A compound inducing a potentiation of both "ends" of the dose - response curve to an agonist is probably acting via an action on the contractile mechanism of the responding cell, whereas a compound acting almost solely on the lower end of the curve is probably acting at a membrane site (Somlyo & Somlyo, 1970). Angiotensin II, in the present experiments, had a greater potentiating action at the lower end of the noradrenaline dose-response curve, thus it is possible that the peptide is acting mainly at the level of the smooth muscle cell membrane.

As the evidence for the lack of a neuronal action of angiotensin II is rather indirect, the next chapter is devoted to a detailed study of a possible inhibiting action of angiotensin II on neuronal uptake of noradrenaline.

5-HT is a well-known non-specific sensitising agent on vascular smooth muscle (de La Lande, Cannell & Waterson (1966); de La Lande, Frewin & Waterson (1967). On the isolated aorta, subthreshold concentrations of 5-HT potentiated responses to exogenous noradrenaline, and this potentiation was blocked by both methysergide and BOL-148. The potentiating action of 5-HT was not antagonised by P-113 showing the specificity of the angiotensin II analogue.

C H A P T E R III

EFFECT OF ANGIOTENSIN II ON NEURONAL UPTAKE OF NORADRENALINE INTO THE RABBIT ISOLATED AORTA

INTRODUCTION

The process of uptake, or reuptake, of noradrenaline into sympathetic nerve endings was recently characterised by Iversen (1967). He found that uptake of either exogenous or endogenous noradrenaline into sympathetic nerve endings was a major mechanism for its removal from the vicinity of adrenergic receptors on smooth muscle.

Cocaine-induced potentiation of the response of various tissues to directly acting sympathomimetic amines is considered to be a result of it's ability to block the neuronal uptake of these amines (Trendelenberg, 1966). Blood vessels show low cocaine induced sensitivity compared with other smooth muscle preparations. Verity (1971) correlated cocaine-induced supersensitivity as a function of the width of the synaptic cleft in a variety of adrenergically innervated tissues, and, as a general rule, suggested that the greater the width of the synaptic cleft, the less the cocaine induced supersensitivity.

Although there is a large synaptic cleft in the rabbit aorta (Bierring & Kobayashi, 1964), several authors have reported an active uptake of

noradrenaline in this tissue which was inhibited by neuronal uptake blocking drugs (Maxwell, Wastilla & Eckhardt, 1966; Nedergaard & Bevan, 1971).

Palaic & Khairallah (1967a) reported that infusions of angiotensin II in concentrations ranging from 8×10^{-9} g/ml to 8×10^{-7} g/ml over a period of one hour significantly inhibited the uptake of noradrenaline into rat brain,. In a later report, Palaic & Khairallah (1967b), angiotensin II at a concentration of 2×10^{-5} g/ml was shown to inhibit noradrenaline uptake into rat brain slices, spleen and aorta. Panisset & Bourdois (1968) have reported a decreased uptake of noradrenaline into cat perfused mesenteric vascular bed on infusion of angiotensin II, and Peach, Bumpus & Khairallah (1969) reported an inhibition of uptake of noradrenaline into the rabbit isolated perfused heart preparation during infusions of angiotensin II. Davila & Khairallah (1970) have defined the uptake mechanism by which angiotensin II acted on isolated rat auricles; they observed an inhibition of uptake of metaraminol by both angiotensin II and ouabain, but whereas the action of ouabain was calcium dependent, that of angiotensin II was not.

Other workers have failed to observe an inhibition of tissue noradrenaline uptake in the presence of angiotensin II. Pals, Fulton & Masucci (1968) reported that the uptake of noradrenaline into the hearts of pithed rats was reduced by cocaine and desipramine

but was unaffected during infusions of angiotensin II. Similarly, Schumann, Starke, Werner & Hellerforth (1970) found that noradrenaline uptake into the rabbit perfused isolated heart was not affected by concentrations of angiotensin II up to 10^{-7} g/ml. Angiotensin II (10^{-7} g/ml), unlike cocaine, failed to prevent the reversal of tyramine tachyphylaxis by noradrenaline in the rat isolated vas deferens (Trager, Kreye & Gross, 1972).

In the rabbit isolated aorta, a catecholamine-uptake blocking action of angiotensin II is a possible mechanism for the observed potentiation of the responses to noradrenaline reported in the previous chapter. The results of other investigators are contradictory, as can be seen from the results reported above. In this chapter, the effect of angiotensin II on uptake of noradrenaline into slices of isolated aorta has been examined.

RESULTS

Effect of angiotensin II on responses to adrenaline, isoprenaline and noradrenaline

The isolated thoracic aorta gave reproducible contractions in response to adrenaline (2×10^{-8} g/ml) noradrenaline (10^{-8} g/ml) and isoprenaline (10^{-6} g/ml). In the presence of angiotensin II (10^{-9} g/ml) responses to each of these compounds were increased by about 100%, the actual figures are given in Fig.24.

Effect of cocaine on responses to noradrenaline, and on the angiotensin II-noradrenaline interaction

Cocaine is known to effectively inhibit noradrenaline uptake in a variety of tissues. Maxwell, Wastilla & Eckhardt (1966) showed that at a concentration of 4.5×10^{-5} g/ml it produced a maximal inhibition of neuronal uptake of noradrenaline in the isolated aorta. Accordingly, this concentration of cocaine was used in four preparations, and Fig.25 illustrates a typical experiment. In the presence of cocaine, the contractions induced by noradrenaline were progressively increased in size. However, when the responses had reached a maximum, the subsequent addition of angiotensin II (10^{-9} g/ml) caused a further increase in the size of the response to noradrenaline. This suggests that angiotensin II is inducing the potentiation of the response to noradrenaline by a different mechanism to that of cocaine. (N.B. Cocaine induced a 50%-200% potentiation of the responses to noradrenaline, and angiotensin II always induced a further 100% potentiation in comparison with control values).

Fig. 24

AGONIST	CONCENTRATION g/ml	POTENTIATION (% CONTROL)	± SE	n
Noradrenaline	10^{-8}	101.8	3.2	6
Isoprenaline	[illegible] 10^{-6}	99.3	2.3	6
Adrenaline	2×10^{-8}	96.4	5.0	5

Fig. 24 A comparison of the effect of angiotensin II (10^{-9} g/ml) on contractions of rabbit isolated aorta induced by noradrenaline (10^{-8} g/ml), adrenaline (2×10^{-8} g/ml) and isoprenaline (10^{-6} g/ml). Potentiations are expressed as percentages of initial responses to each agonist.

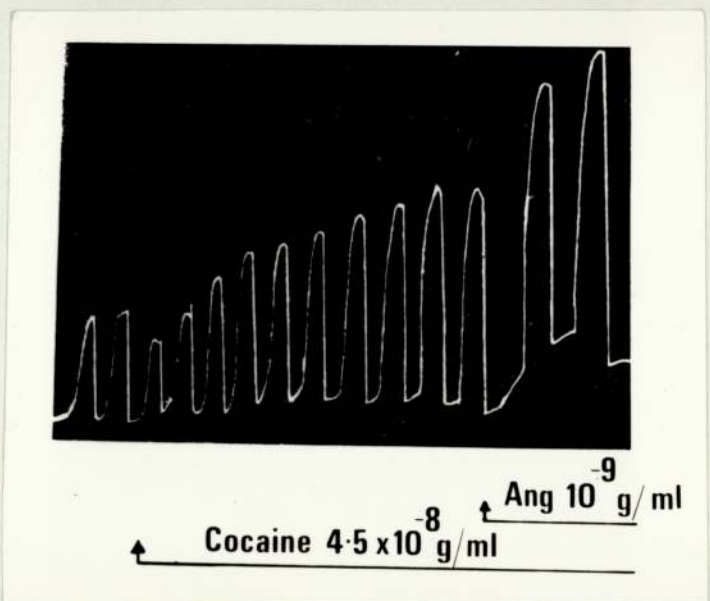

Fig. 25 Rabbit isolated aortic strip.
Contractions to noradrenaline (10^{-8} g/ml) were increased in the presence of cocaine (4.5×10^{-5} g/ml) and further enhanced when angiotensin II was added to the bath.

Effect of guanethidine on responses to noradrenaline, and on the angiotensin II noradrenaline interaction

Responses to noradrenaline (10^{-8} g/ml) were potentiated in each of four preparations on exposure of the tissue to guanethidine (5×10^{-5} g/ml) at a concentration having a maximal effect on catecholamine uptake in this tissue (Maxwell et al, 1962). During exposure of the tissue to guanethidine, the basal tone of the tissue increased until the base-line was at a height equivalent to roughly double the original size of the control response to noradrenaline. This made the preparation difficult to work with; when angiotensin II was added to the organ bath at a concentration of 10^{-9} g/ml there was a further increase in the size of the response to noradrenaline. On average, angiotensin II increased the size of the response by a further 100% in comparison with control values, i.e. guanethidine did not alter the percentage potentiation induced by angiotensin II.

Effect of bethanidine on responses to noradrenaline, and on the angiotensin II noradrenaline interaction

Responses to noradrenaline (10^{-8} g/ml) were potentiated to a maximum of 100% in each of four preparations tested on the addition of bethanidine (5×10^{-5} g/ml) to the organ bath. This concentration of bethanidine had been found to be an effective concentration to block the effect of low frequency nerve stimulation in previous experiments (Chap.II). Unlike guanethidine, the presence of bethanidine

induced no alteration in tone of the tissue. Addition of angiotensin II (10^{-9} g/ml) induced a further potentiation of the response to noradrenaline, on average 100% above the control value. A typical experiment is illustrated in Fig.26. Again this appears evidence that angiotensin II is not acting by an inhibition of neuronal uptake.

Effect of DMI on responses to noradrenaline

At concentrations below 10^{-6} g/ml, DMI produced no effect on the size of the responses to noradrenaline, and no effect on the percentage potentiation of responses to noradrenaline by angiotensin II. Above this concentration of DMI, responses to noradrenaline were reduced. This apparent α- adrenergic receptor blocking action of DMI has previously been observed in the rabbit aorta (Toda, 1971). Due to this interference by the α-blocking action of DMI, no further experiments were performed using this compound.

Tyramine tachyphylaxis and its reversal

Burn & Rand (1958) first demonstrated the reversal of tyramine tachyphylaxis by noradrenaline. Using reserpinised spinal cats, they observed only small rises in blood pressure on injection of tyramine; after an infusion of noradrenaline, the responses to tyramine were greatly enhanced. Repetitive doses of tyramine induced a tachyphylaxis to its constrictor

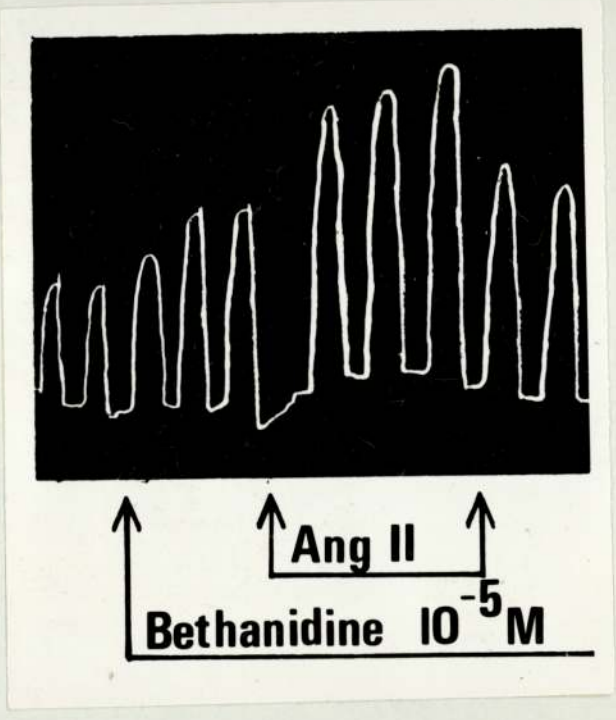

Fig. 26 Rabbit aortic strip;
contractions to noradrenaline (10^{-8} g/ml) were increased in the presence of bethanidine (10^{-5} M) and further enhanced when angiotensin II (10^{-9} g/ml) was added to the bath.

effect, and a further infusion of noradrenaline reversed the tachyphylaxis. Furchgott, Kirpekar, Rieker & Schwab (1963) reported similar experiments using the isolated thoracic aorta of the rabbit. They found that concentrations of tyramine of 10^{-6} g/ml and below induced responses by an indirect action via release of noradrenaline from adrenergic nerve endings, whereas concentrations above 10^{-6} g/ml induced contractions by a direct action on vascular smooth muscle.

(a) Reversal of tachyphylaxis Repetitive contact of the tissue with tyramine (10^{-6} g/ml) induced a marked tachyphylaxis (an average 85% reduction of response) to the contractor action after 8 to 10 doses. On incubation of the tissue with noradrenaline (4×10^{-6} g/ml) for 30 min., then washout and a rest period of 15 min., responses to tyramine showed a significant degree of recovery (results summarized in Fig.27b). Repetition of this procedure, but not exposing the tissue to noradrenaline, resulted in no reversal of the tachyphylaxis (results summarized in Fig.27a).

(b) Effect of cocaine Using four preparations, tachyphylaxis to tyramine was again induced by repeated application of the drug. Cocaine (4.5×10^{-5} g/ml) was added to the organ bath, and 10 min. later noradrenaline was added, and the tissue incubated in this mixture for a further 30 min.

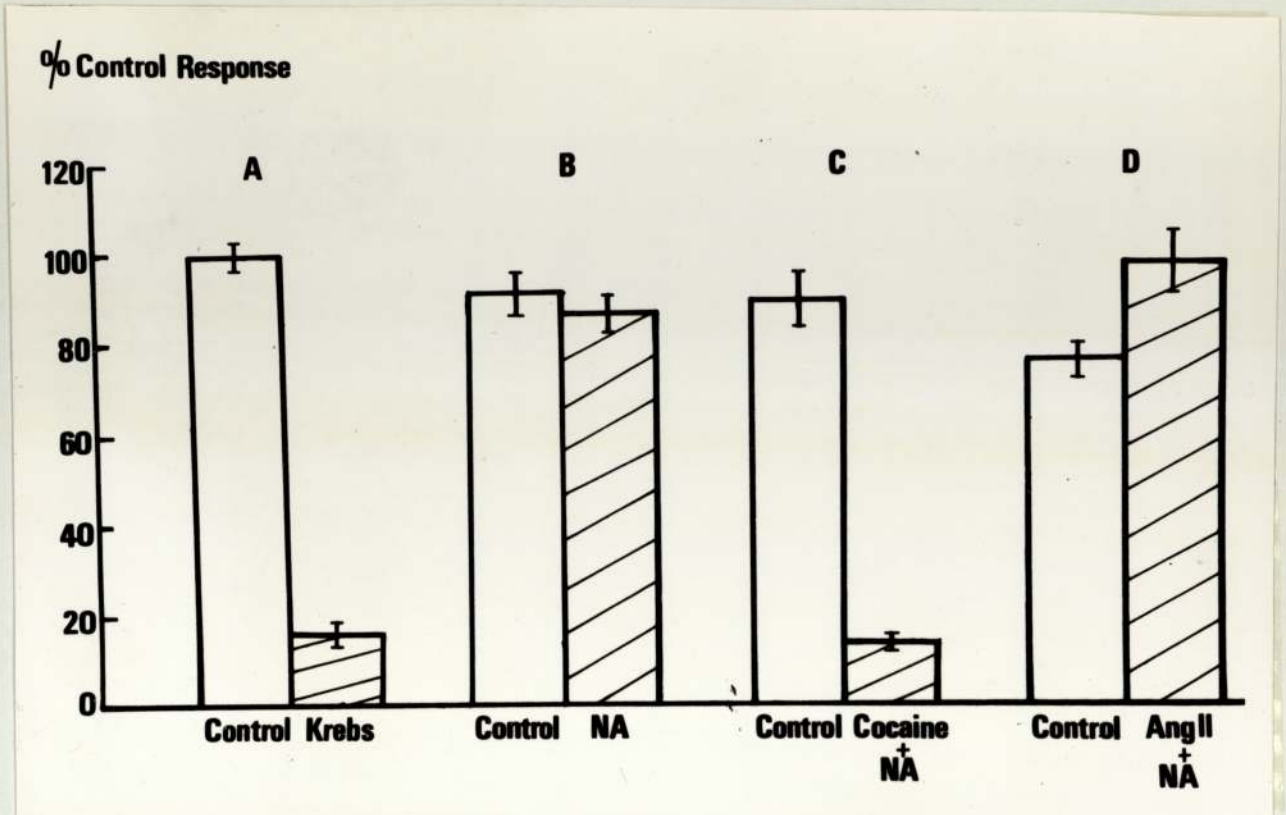

Fig. 27 Rabbit isolated aortic strips

Histogram summarising the effect of various procedures on tachyphylaxis to tyramine. The method attaining this tachyphylaxis is described in the text.

In (a) Open column represents control response to tyramine (10^{-6}g/ml) expressed as 100% and the hatched column represents tyramine response after induction of tachyphylaxis, as a percentage of control.

(b) Reversal of tyramine tachyphylaxis after incubation for 30 min. with noradrenaline (4×10^{-6}g/ml)

(c) Prevention of noradrenaline reversal of tyramine tachyphylaxis by noradrenaline (4×10^{-6}g/ml) in the presence of cocaine (4.5×10^{-5}g/ml).

(d) Lack of effect of angiotensin II (10^{-9}g/ml) on reversal of tyramine tachyphylaxis by noradrenaline (4×10^{-6}g/ml).

On washout of the drugs, and a rest period of 15 min., the minute response evinced on the addition of tyramine indicated that no reversal of the tyramine tachyphylaxis had occurred, a summary of the results is shown in Fig.27(c)

(c) Effect of angiotensin II Using four preparations, tachyphylaxis to tyramine was again induced by repeated exposure of the tissue to the drug. The tissue was then exposed to angiotensin II at a concentration of 10^{-9} g/ml for 10 min. before addition of noradrenaline (4×10^{-6} g/ml) and the tissue was incubated in this mixture for a further 30 min. On washout of the drugs and a rest period of 15 min., exposure of the tissue to tyramine induced a contraction equal in size to that of the controls. A summary of the results is shown in Fig.27(d).

Angiotensin II, unlike cocaine, did not prevent uptake of noradrenaline at the concentration in contact with the tissue, and hence did not prevent the reversal of tyramine tachyphylaxis. A similar lack of inhibition of noradrenaline reversal of tyramine tachyphylaxis in the presence of angiotensin II has been reported by Trager et al (1972) using the rat isolated vas deferens.

Radioactive noradrenaline uptake measurements

The results of the experiments reported in this chapter suggest that angiotensin II at the concentration of 10^{-9} g/ml, inducing a potentiation of responses to noradrenaline, did not inhibit neuronal uptake of noradrenaline. However, in each case, the results are of an indirect nature, therefore, measurements of the actual uptake of noradrenaline and the effect of angiotensin II on this uptake were carried out using radioactive noradrenaline.

(a) Effect of mode of death on uptake of ^{14}C-NA

Rabbits were killed by one of three methods; a blow on the neck followed by exsanguination, air embolism, or an overdose of phenobarbitone. An examination of the degree of uptake of ^{14}C-NA into isolated aortae taken from each of these groups of rabbits revealed no significant difference between them. In the experiments described in the rest of this chapter, rabbits were killed by a blow on the neck followed by exsanguination.

(b) Effect of cocaine on uptake of ^{14}C-NA

The concentration of radioactive noradrenaline used in these experiments was 6×10^{-8} g/ml (2×10^{-7}M) Nedergaard & Bevan (1971) found that at concentrations above 10^{-6} M, there was an extra-neuronal uptake of noradrenaline in the isolated aorta. To ensure that the radioactivity measured was in fact of neuronal rather than extraneuronal origin, the effect of cocaine

on the measured activity was examined. It was found that cocaine, in a concentration of 4.5×10^{-5} g/ml, induced an 83% inhibition of uptake of ^{14}C-NA into the tissue, after 30 min. incubation of the radioactive noradrenaline with the tissue, as can be seen in Fig.28. This figure is in close agreement with that of Maxwell et al (1968) using cocaine under the same conditions with the same tissue, and strongly suggests that this measured radioactivity detected after incubation of the aorta with ^{14}C-NA is due to a concentration of radioactive noradrenaline in the sympathetic nerve endings in the tissue by a cocaine sensitive method.

(c) Effect of angiotensin II on uptake of ^{14}C-NA

Isolated aortic rings were incubated for 10 min. with angiotensin II at a concentration of either 10^{-9} or 10^{-6} g/ml before addition of ^{14}C-NA. Strips were incubated for up to one hour with the ^{14}C-NA, and then the radioactivity contained in them was measured as described in the METHODS section. As can be seen from the results expressed graphically in Fig.29 angiotensin II at either concentration had no significant effect on the neuronal uptake of radioactive noradrenaline.

Palaic & Khairallah (1967b) suggested that angiotensin II may be broken down rapidly by tissues in vitro, and hence should be given simultaneously with noradrenaline when uptake measurements are made. It can be seen from Fig.30 that, over a 30 min. period,

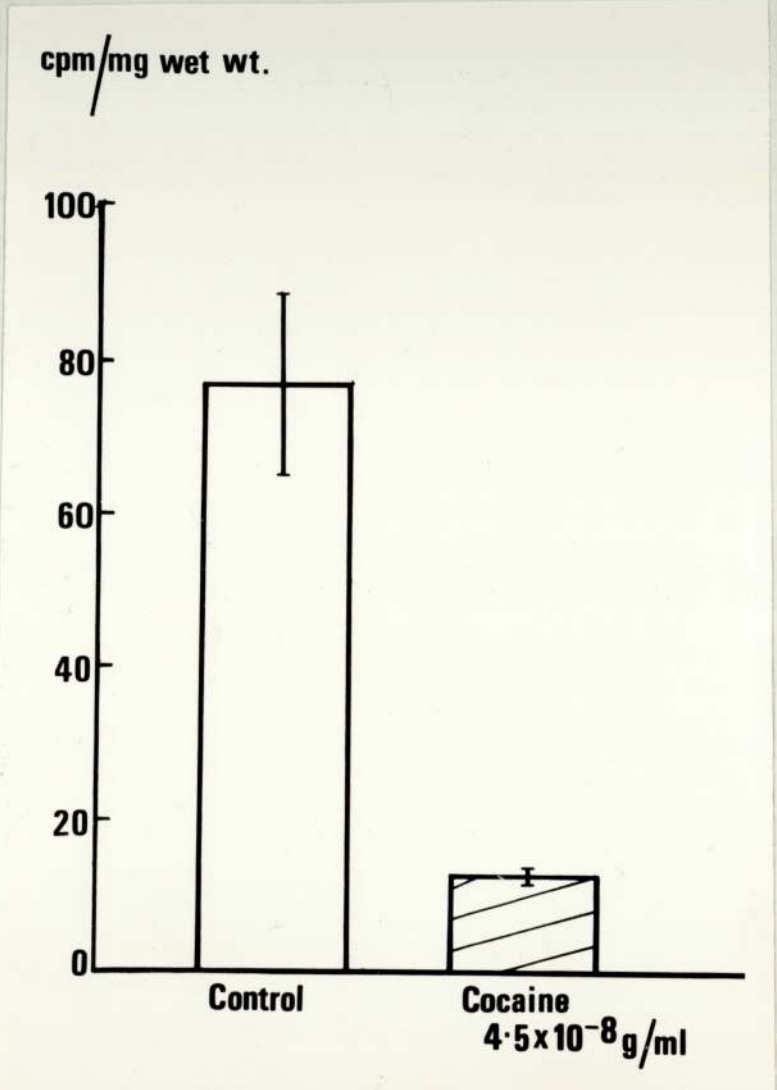

Fig. 28 Uptake of ^{14}C-NA into rabbit isolated aortic rings over a 30 min. period, and the uptake-inhibiting action of cocaine (4.5×10^{-5} g/ml).

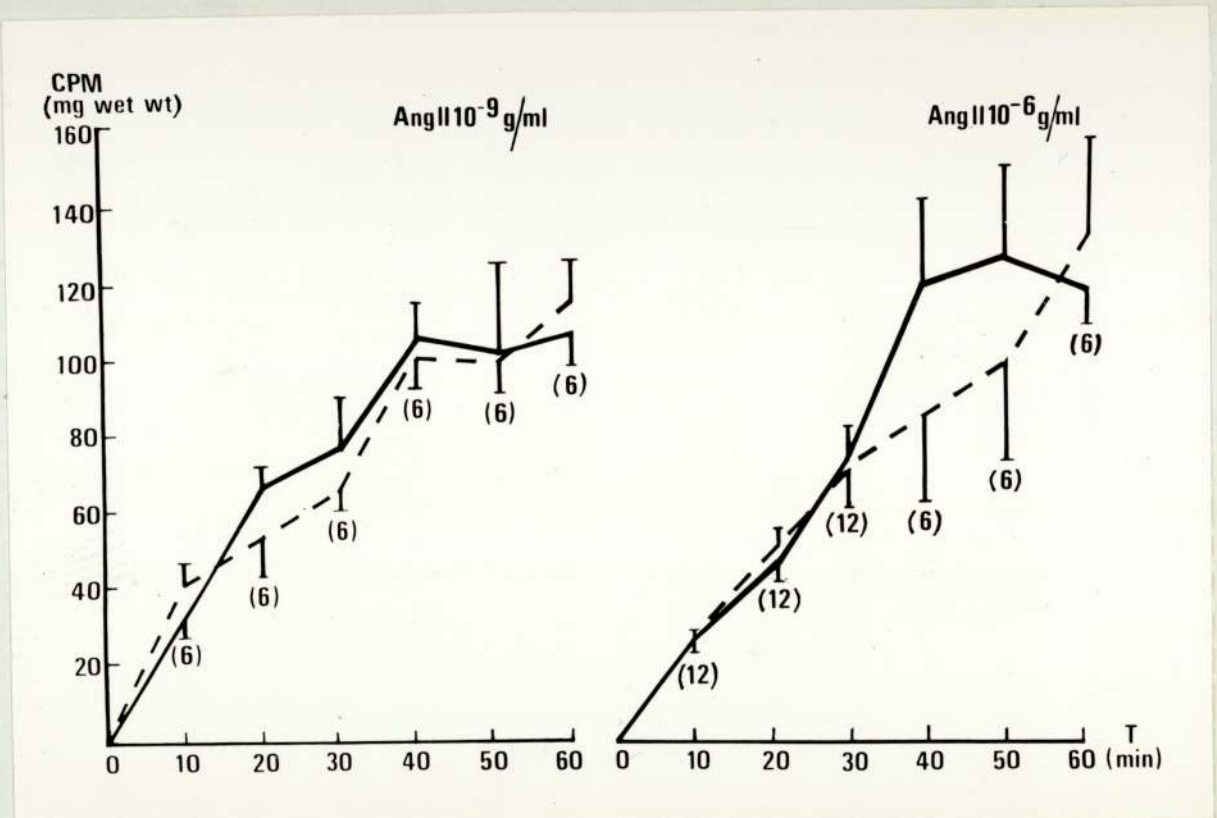

Fig. 29 Influence of angiotensin II on uptake of ^{14}C - labelled d,l - noradrenaline into isolated rings of rabbit aorta at 37°C. Continuous lines represent control experiments and broken lines incubations in the presence of angiotensin II. Experimental procedure as described under methods.

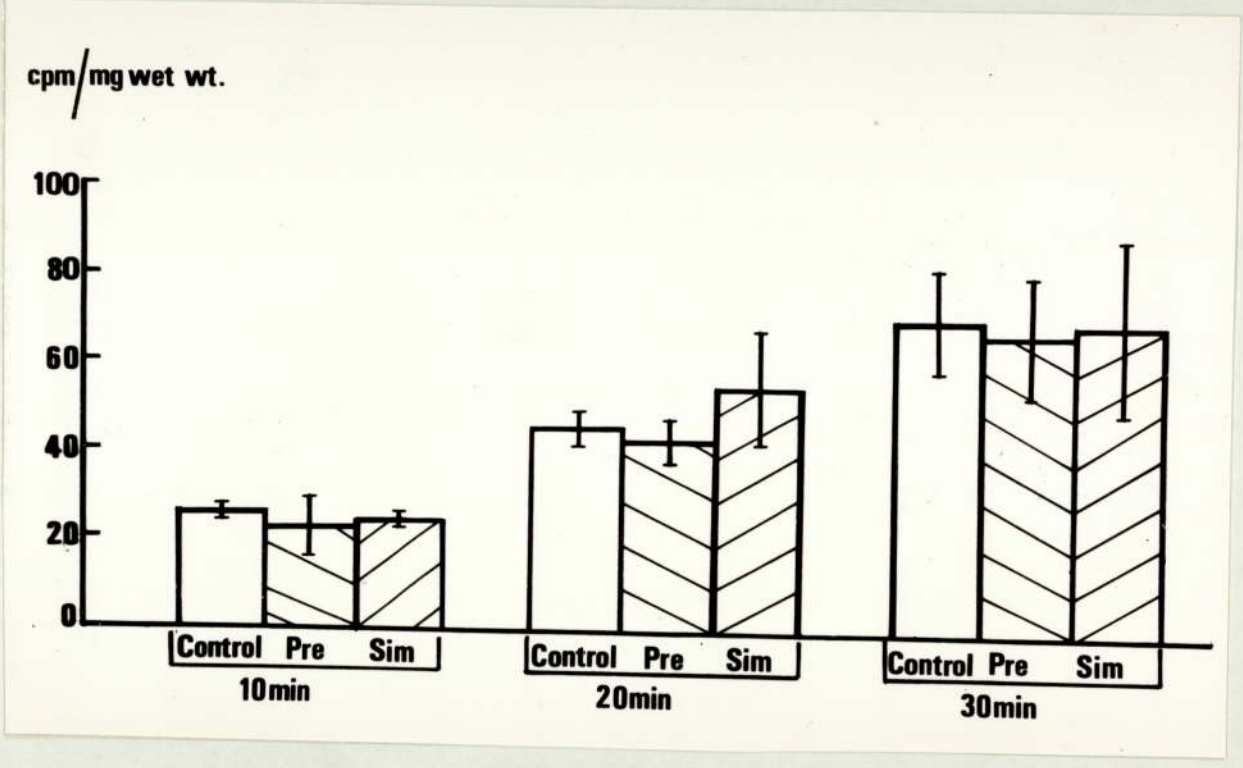

Fig. 30 Influence of time of addition of angiotensin II to the tissue on the uptake of ^{14}C - labelled d,l - noradrenaline into isolated rings of rabbit aorta at 37°C. Control is no angiotensin II, Pre = angiotensin II given 10 min. before noradrenaline, and sim is angiotensin II and noradrenaline given simultaneously.

whether angiotensin II was given 10 min. before the ^{14}C-NA or simultaneously, it still had no significant effect in comparison with control values (at a concentration of 10^{-6} g/ml.)

(d) Effect of angiotensin II on wet weight of tissues

All measurements of uptake of radioactivity into isolated aortic rings were expressed as cpm/mg wet weight of tissue. As angiotensin II had no significant effect on the uptake of noradrenaline into the tissue, it was important to ensure that angiotensin II had no effect on the actual wet weight of the tissue. Fig.31 gives a table of results showing that, after incubation of tissues with angiotensin II at either 10^{-9} or 10^{-6} g/ml., there was no significant difference from control wet weights. Therefore, a possible action of angiotensin II on uptake of ^{14}C-NA was not being masked by the peptide altering the wet weight of the tissue.

Discussion

Iversen (1967) reported that the affinity of the amine-uptake process in the sympathetic nerve ending is greater for noradrenaline than adrenaline, and greater for adrenaline than isoprenaline. In fact, Callingham & Burgen (1966) found no specific uptake of isoprenaline into the sympathetic nerve ending. The equal degree of potentiation of all three sympathomimetic amines in the presence of angiotensin II suggests that the polypeptide is not acting on the

Fig. 31

	W_1 ± S.E.	W_2 ± S.E.	N	T test (W_1 & W_2)
CONTROL	7.48 ± 0.46	7.35 ± 0.25	10	not significant
ANGIOTENSIN II 10^{-9} g/ml	8.43 ± 0.39	8.41 ± 0.29	10	not significant
ANGIOTENSIN II 10^{-6} g/ml	8.50 ± 0.65	7.77 ± 0.59	10	not significant

Fig. 31 Effect on wet weight of aortic rings of incubation for 1 hr. at 37°C with two concentrations of angiotensin II. W_1 - initial weight; W_2 - weight after incubation. All weights are in mg.

neuronal amine-uptake process, at least in the potentiation of responses to isoprenaline.

Turker & Karahuseyinoglu (1968) found that responses of the rabbit isolated aorta to tyramine were totally abolished in the presence of cocaine at a concentration of 10^{-5} g/ml. Toda (1971) found that concentrations of 3×10^{-7} to 10^{-6} g/ml of cocaine caused the maximum potentiation of the responses of rabbit isolated aorta to noradrenaline. However, Maxwell, Daniel, Sheppard & Zimmerman (1962) used concentrations of 3×10^{-5} g/ml and above to induce a maximum potentiation of the responses to noradrenaline in this tissue, and Maxwell, Wastilla & Eckhardt (1966) found that at a concentration of 4.5×10^{-5} g/ml cocaine induced a maximum inhibition of uptake of radioactive noradrenaline into the rabbit aorta, an 85% reduction of uptake, while concentrations above this caused a further potentiation of the response without inducing any further inhibition of neuronal uptake. Nedergaard & Bevan (1971) found that on raising the concentration of cocaine from 3×10^{-5} to 3×10^{-4} g/ml there was a progressive increase in inhibition of uptake of noradrenaline into the medial coat of the rabbit aorta, indicating a possible non-specific action of cocaine, as the sympathetic nerves in the rabbit aorta are located at the adventitio-medial junction (Maxwell et al, 1968).

Using a maximally effective neuronal uptake blocking concentration of cocaine (Maxwell, 1966) responses to noradrenaline were potentiated by 50 to 200%, and contact with angiotensin II caused a further 100% (in comparison with controls) enhancement of the responses to noradrenaline. This suggests that either angiotensin II does not act in this tissue by inhibiting neuronal uptake of noradrenaline, or that as cocaine does not induce a 100% inhibition of uptake the combination of cocaine with angiotensin II may induce a 100% blockade, and hence responses to noradrenaline were further potentiated. That the concentration of cocaine used was a maximally effective concentration in this tissue was confirmed by the ^{14}C-NA uptake experiments, where an 83% inhibition of uptake was observed.

The presence of either guanethidine or bethanidine failed to prevent a further potentiation of the responses to noradrenaline on contact of the tissue with angiotensin II. DMI, however, displayed an α-receptor blocking action so that it could not be used in the elucidation of the mechanism of action of angiotensin II.

The reported observations of Furchgott et al (1963) were repeated in the present experiments; cocaine prevented the reversal of tyramine tachyphylaxis when present throughout the incubation of the tissue with

noradrenaline. However, angiotensin II did not prevent the reversal of tyramine tachyphylaxis when incubated simultaneously with noradrenaline. These results strongly suggest that angiotensin II, at the concentration given does not prevent uptake of noradrenaline into the sympathetic nerve endings of the rabbit isolated aorta.

An active, neuronal uptake of noradrenaline has been reported to occur in the rabbit isolated aorta by both Maxwell et al (1968) and Nedergaard & Bevan (1971). The latter authors reported that at concentrations of radioactive noradrenaline greater than 10^{-6} M, medial tissue may concentrate the amine by a specific transport process. Gillespie & Hamilton (1967) and Avakian & Gillespie (1968) demonstrated an intracellular accumulation of noradrenaline by arterial smooth muscle. Thus to avoid the non-neuronal uptake of noradrenaline into the aorta, a concentration lower than 10^{-6} M was used in the present series of experiments. However, due to practical considerations of the actual counting of the radioactivity in the tissue, a concentration not lower than 2×10^{-7} M of noradrenaline was used; i.e. this concentration was lower than that needed to stimulate a medial catecholamine-specific transport process, while enabling sufficient disintegrations per minute to be counted and so avoid an invalid set of results due to large inaccuracies in measurement.

Angiotensin II at either 10^{-9} or 10^{-6} g/ml had no significant effect on the uptake of ^{14}C-NA into isolated aortic rings, nor did it affect the wet weight of the tissue. Palaic & Khairallah (1967b) gave noradrenaline and angiotensin II simultaneously, and stated that, due to the rapid breakdown of angiotensin II in vitro, this was the only valid experimental procedure. In the present experiments whether angiotensin II was given either 10 min. before or simultaneously with noradrenaline, it still had no significant effect on uptake of noradrenaline. Khairallah et al (1966) noted that heavy metal chelating compounds, such as EDTA, inhibit the activity of tissue angiotensinases. In the series of experiments involving the use of radioactive noradrenaline, EDTA was present throughout the experiments, hence it is unlikely that there was any inactivation of the angiotensin II.

Throughout the experiments involving measurement of radioactivity in the aorta, the ^{14}C-NA uptake into the whole tissue was measured, i.e. uptake into adventitial and medial layers was not separated. In theory, angiotensin II could be blocking neuronal uptake, but at the same time increasing medial uptake of noradrenaline, thus masking the effect of the inhibition. This is unlikely for several reasons:-

(i) The tissue was thoroughly washed after incubation, to remove as much extracellular noradrenaline as possible.

(ii) An increased intracellular uptake of noradrenaline into medial smooth muscle cells would reduce the concentration of agonist at the α-receptor, hence one would expect a decreased, rather than an increased, response to noradrenaline in the presence of angiotensin II.

(iii) Cocaine inhibits the uptake of noradrenaline into the sympathetic nerve ending; this reduced uptake was measured in this series of experiments, i.e. although neuronal uptake was decreased, there was no corresponding medial increase in uptake of noradrenaline.

CHAPTER IV

THE ROLE OF CALCIUM IN THE POTENTIATING ACTION OF ANGIOTENSIN II ON THE RABBIT ISOLATED AORTA

INTRODUCTION

The parameters of the potentiation of noradrenaline by angiotensin II have been described in Chapter II; from the greater action of angiotensin II in the lower region of the dose-response curve it appears that a membrane-effect is involved, rather than an action on the contractile mechanism itself. The results of the investigation described in Chapter III indicate that angiotensin II is not acting on the aorta by an inhibition of neuronal uptake of noradrenaline.

Angiotensin II does have other actions which could possibly contribute to the potentiation. Amongst these are a release of neuronally-bound noradrenaline (Liebau, Distler & Wolff, 1965), a post synaptic interaction with noradrenaline at the level of the vascular α-receptor (Pals & Fulton, 1968) and an alteration of transcellular or intracellular calcium fluxes (Shibita, Carrier & Frankenheim, 1968; Angles d'Auriac, Baudouin & Meyer, 1972). The present chapter includes an investigation into the involvement of these three factors in the potentiation of noradrenaline by angiotensin II, and also the role of calcium ions in the potentiation of noradrenaline by 5-HT.

RESULTS

Effect of angiotensin II on responses to other spasmogens

Since the enhancement of noradrenaline responses by angiotensin II appeared to be independent of effects on neuronal noradrenaline uptake, the possible effects of the peptide on responses to other spasmogens in this tissue were examined. Accordingly, suitable concentrations of five spasmogens: potassium chloride, acetylcholine, histamine, isoprenaline and 5-HT to give similar sized submaximal contractions to noradrenaline (10^{-8} g/ml) were added to aortic strips in the presence and absence of angiotensin II (10^{-9} g/ml). The results are summarised in Fig.32. The responses to all agonists were increased in the presence of angiotensin II to an almost identical extent. As mentioned in Chapter II, the depressant action of constrictor concentrations of angiotensin II on responses to noradrenaline also occurs on responses to potassium chloride, 5-HT, acetylcholine and histamine.

Effect of reserpinisation on the angiotensin II-induced potentiation of noradrenaline and 5-HT

Rabbit thoracic aortae exposed to reserpine in vitro exhibit a non-specific decrease in reactivity to spasmogens after prolonged incubation (Hong & Rodriguez, 1965). It was found that the solvent was largely responsible for this decreased reactivity. For experiments requiring catecholamine-depleted aortae, blood vessels were taken from reserpine pretreated animals.

Fig. 32

AGONIST	CONC$^{n.}$ (g/ml)	POTENTIATION (% CONTROL)	S.E. (+ or -)	n
KCl	1.17×10^{-3}	100.0	1.1	5
NA	10^{-8}	101.8	3.2	6
HISTAMINE	1.20×10^{-7}	97.0	1.8	6
ACh	4.0×10^{-6}	94.0	3.1	5
ISOPRENA-ALINE	4.0×10^{-6}	99.3	2.3	6
5HT	4.0×10^{-8}	97.0	1.2	5

Fig. 32 Effect of angiotensin II (10^{-9} g/ml) on contractions of rabbit isolated aorta induced by a variety of agonists. Potentiations are expressed as percentages of initial responses to each agonist (i.e. 100% potentiation is equivalent to a doubling of the response).

Preparations were taken from seven rabbits which had been pretreated with reserpine; five of these showed an enhanced sensitivity to the spasmogenic activity of angiotensin II, whilst the sensitivity of the other two was within the normal range. Sakurai & Hashimoto (1965) made a similar observation of increased or unaltered sensitivity to angiotensin II on vascular smooth muscle from reserpinised rabbits. In the tissues exhibiting an enhanced sensitivity to angiotensin II, a concentration of 2×10^{-10} g/ml was found to be subthreshold to cause a contraction, but caused a similar degree of enhancement of responses to noradrenaline (10^{-9} g/ml) and 5-HT (4×10^{-8} g/ml) as did a concentration of 10^{-9} g/ml in control preparations. In the other two preparations exhibiting normal reactivity to angiotensin II a concentration of 10^{-9} g/ml enhanced 5-HT and noradrenaline responses to a similar degree as in control preparations. These results would suggest that acute release of endogenous noradrenaline by angiotensin II is unlikely to be involved in the angiotensin II-induced potentiation of responses to other vascular spasmogens. The results of a typical experiment are seen in Fig.33.

Effect of SKF-525A on angiotensin II - potentiation of noradrenaline

SKF-525A has been reported to be a specific inhibitor of potassium-induced contractions of the rabbit isolated aorta (Kalsner, Nickerson & Boyd, 1970)

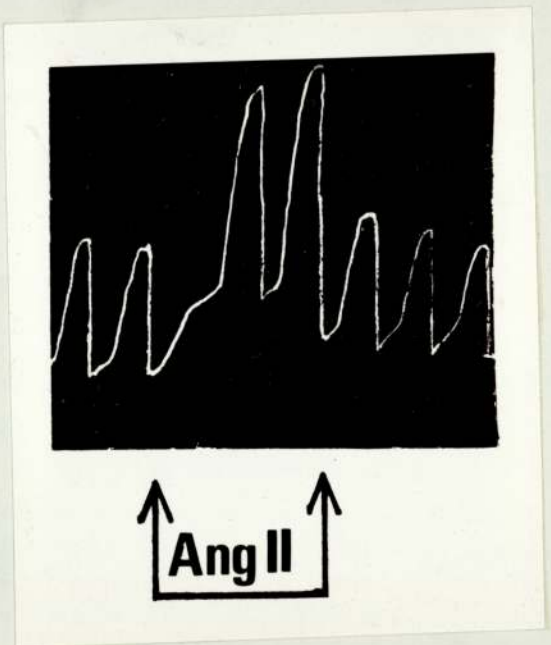

Fig. 33 Rabbit isolated aortic strip;
aorta taken from reserpinised rabbit responds to noradrenaline (10^{-9} g/ml) and responses are potentiated in the presence of angiotensin II (10^{-9} g/ml).

possibly by an effect on transmembrane calcium transport. In all four experiments in which SKF 525A was used, responses to noradrenaline were significantly inhibited but as can be seen from the results summarised in Fig.34, the percentage potentiation of responses to noradrenaline by angiotensin II was not affected. The antagonist itself had no effect on baseline.

As this, possibly somewhat specialised, transport of membrane calcium did not appear to be involved in the potentiating action of angiotensin II, the involvement of extracellular calcium was investigated in a more direct manner; the tissue was incubated in calcium-free medium.

Incubation in Calcium-free medium

(a) Effect on potentiation of noradrenaline by 5-HT

The potentiation of responses to noradrenaline by 5-HT was totally blocked in each of four preparations after incubation of the tissue in calcium-free medium for two hours. The results are summarised in Fig.35. Initially, it may be proposed that this calcium-dependent action of 5-HT may be either post or pre-synaptic, as noradrenaline release is calcium-dependent (Blaustein, Johnson & Needleman, 1972). As 5-HT is known to release noradrenaline from sympathetic nerve endings, it may be that 5-HT is acting in a similar manner to indirectly acting sympathomimetic amines, described by Khairallah et al (1966b). However, as described in Chapter VI, aortae taken from reserpinised

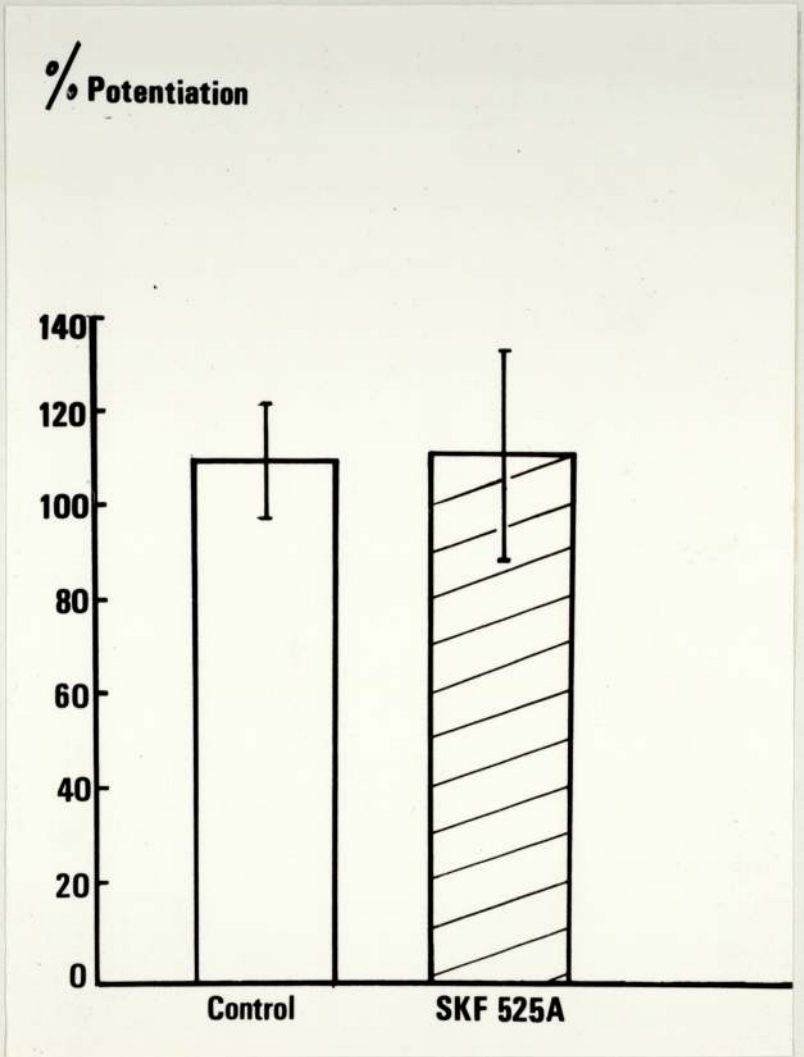

Fig. 34 Incubating rabbit aortae in SKF 525A (10^{-5} g/ml) did not alter the % potentiation of noradrenaline by angiotensin II

Open column: % potentiation of noradrenaline (10^{-8} g/ml) by angiotensin II (10^{-9} g/ml)

Hatched column: % potentiation of NA (10^{-8} g/ml) by angiotensin II (10^{-9} g/ml) with SKF 525A. N = 4.

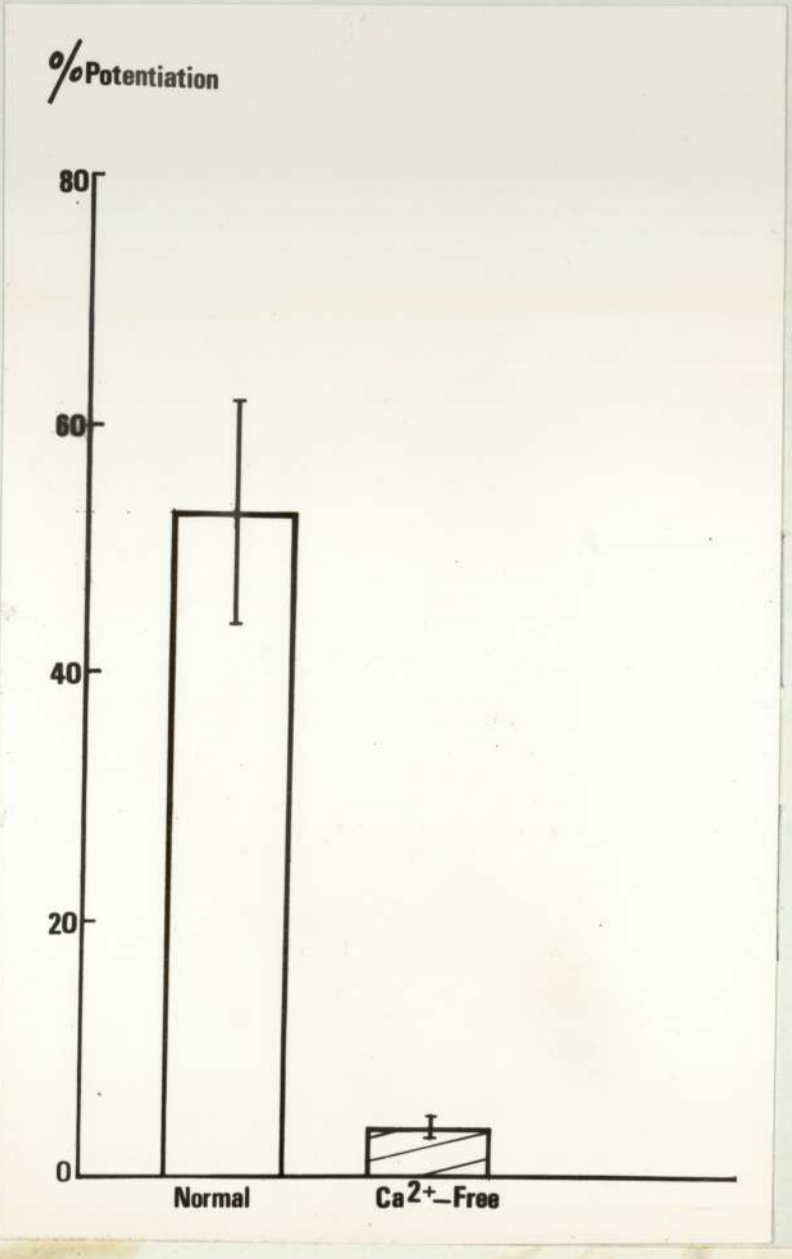

Fig. 35 The potentiation of responses to noradrenaline by 5-HT (1.6×10^{-9} g/ml) was significantly reduced on incubation of rabbit isolated aorta with Ca^{2+}-free medium;
Open column: % potentiation of NA (10^{-8} g/ml) by 5-HT (1.6×10^{-9} g/ml),
Hatched column: % potentiation of NA (4×10^{-8} g/ml) by 5-HT (1.6×10^{-9} g/ml) in Ca^{2+}-free medium. N=4.

rabbits are still sensitive to the potentiating action of 5-HT, therefore, it would appear that this effect is post-synaptic. It may, therefore, be proposed that 5-HT induces a sensitivity to the action of noradrenaline by a post-synaptic mechanism involving extracellular calcium, possibly by increasing the influx of extracellular calcium during the response to noradrenaline.

(b) Effect on potentiation of noradrenaline by angiotensin II

Responses to noradrenaline were reduced by a mean of 75% in comparison with controls in each of six preparations exposed to calcium-free bathing medium, confirming the observation of Hudgins & Weiss (1968). The percentage potentiation by angiotensin II (10^{-9} g/ml) of responses to noradrenaline was not significantly affected by this treatment, but the reduction of responses to noradrenaline seen in the presence of contractor concentrations of angiotensin II in normal Krebs solution was converted to a potentiation. These results are expressed in the form of a histogram in Fig.36. Low concentrations of angiotensin II which had caused a contraction of the aorta in normal bathing medium, induced no contraction in calcium-free medium. It appears unlikely, therefore, that calcium ions have a direct role in the observed potentiation of responses to noradrenaline by angiotensin II.

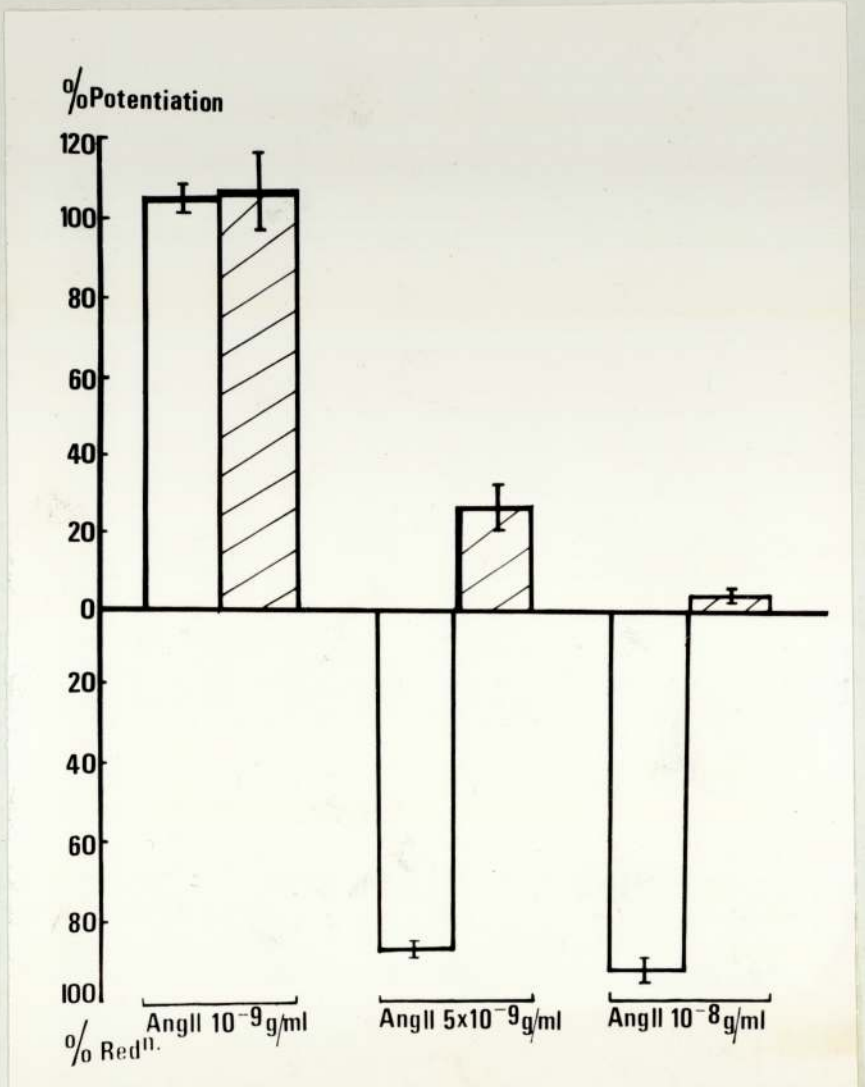

Fig. 36 Effect of increasing concentrations of angiotensin II on contractions of aortic strips to noradrenaline. Open columns represent responses to noradrenaline (2×10^{-8} g/ml) in normal Krebs solution and cross-hatched columns in Ca^{2+}-free Krebs (noradrenaline 8×10^{-8} g/ml). The results are expressed as a percentage of the initial control responses to noradrenaline in each medium. Each column is the mean of 6 experiments; vertical bars are S.E. of mean.

However, Somlyo & Somlyo (1970) have pointed out that by exposing the tissue to "Ca^{++} free" medium, the actual (extracellular) calcium may not be markedly affected; therefore experiments were repeated using calcium-free Krebs containing the calcium-chelating compound EDTA. However the ommission of calcium from the medium blocked the 5-HT induced potentiation of responses to noradrenaline.

(c) Effect of calcium-free plus EDTA-containing medium on the angiotensin II potentiation of noradrenaline

In normal medium, exposure of the tissue to EDTA had a slight potentiating action on the responses to noradrenaline, but did not affect the percentage potentiation of noradrenaline by angiotensin II. However, in each of four experiments, exposure of the tissue to calcium-free + EDTA (0.01mM) medium so strongly reduced responses to noradrenaline that a concentration of 10^{-6} g/ml of noradrenaline was used to induce repeatable responses, instead of the usual 10^{-8} g/ml in standard Krebs solution. On addition of angiotensin II (10^{-9} g/ml) there was no potentiation of the response to noradrenaline. The results are expressed as a histogram in Fig.37. Thus it appears that the potentiation of noradrenaline by angiotensin II does, in fact, involve calcium, but that this calcium is in either a less accessible or more strongly bound form than the calcium required for the potentiation of responses to noradrenaline by 5-HT. However, observations made by Daniel (1966)

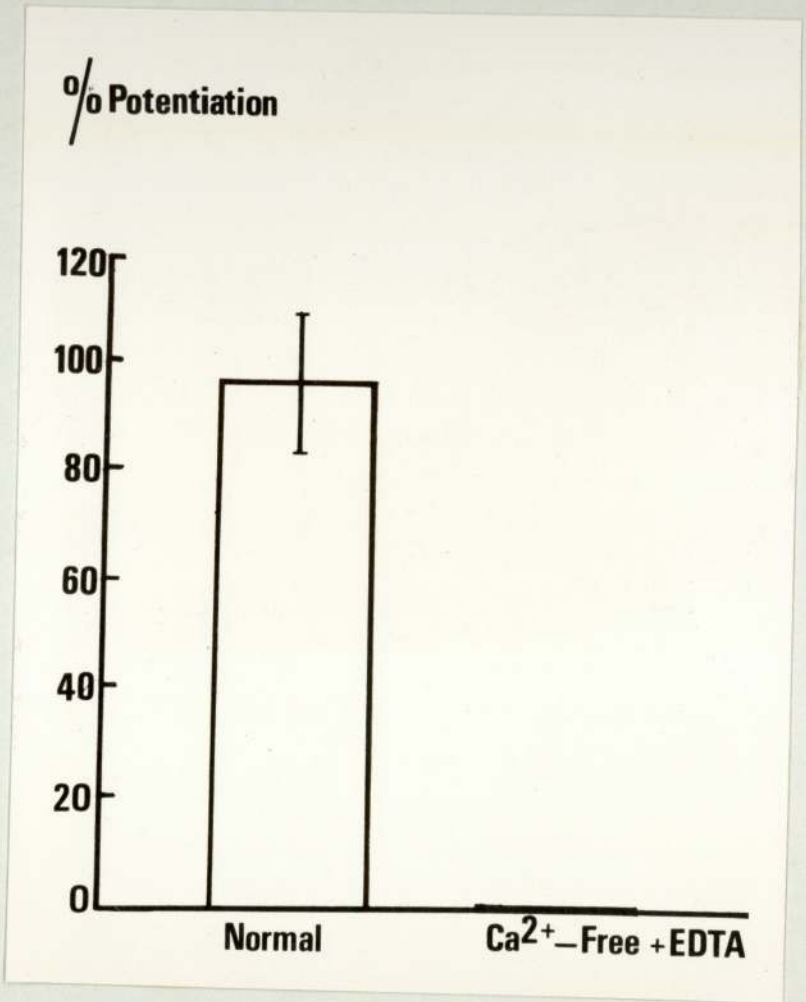

Fig. 37 Effect of incubating rabbit isolated aorta in Ca^{++}-free + EDTA Krebs on the potentiation of NA (10^{-8} g/ml) by angiotensin II. The open column is the % potentiation in normal Krebs, and the hatched column that in Ca^{++}-free + EDTA Krebs (noradrenaline 10^{-6} g/ml). N = 4.

offer an alternative possibility; he found, using the rabbit isolated aorta, that on incubation of the tissue in calcium-free plus EDTA-containing medium the membrane sodium-pump was blocked, therefore, it may be that the potentiation of noradrenaline by angiotensin II, in fact, does not directly involve extracellular calcium, but does involve an action at the sodium pump. The total removal of extracellular calcium is a rather severe procedure. However, if a calcium-free plus EDTA-containing medium inhibits the potentiation of noradrenaline by angiotensin II, and this effect is due directly to a decrease in the concentration of external calcium, then an increase in extracellular calcium would be expected to induce a greater percentage potentiation of noradrenaline by angiotensin II in comparison with normal Krebs.

Effect of increased calcium on the potentiation of noradrenaline by angiotensin II

In each of five experiments, doubling the concentration of calcium chloride in the bathing medium had no significant effect on the response to noradrenaline. However, on addition of angiotensin II, four of the preparations exhibited no potentiation of the response to noradrenaline, whereas the fifth gave a normal percentage potentiation; the results are summarised in Fig.38. A raised external calcium level would be expected to stabilize the membrane, but responses to noradrenaline were not reduced. This possible role of the membrane suggests that angiotensin II may act

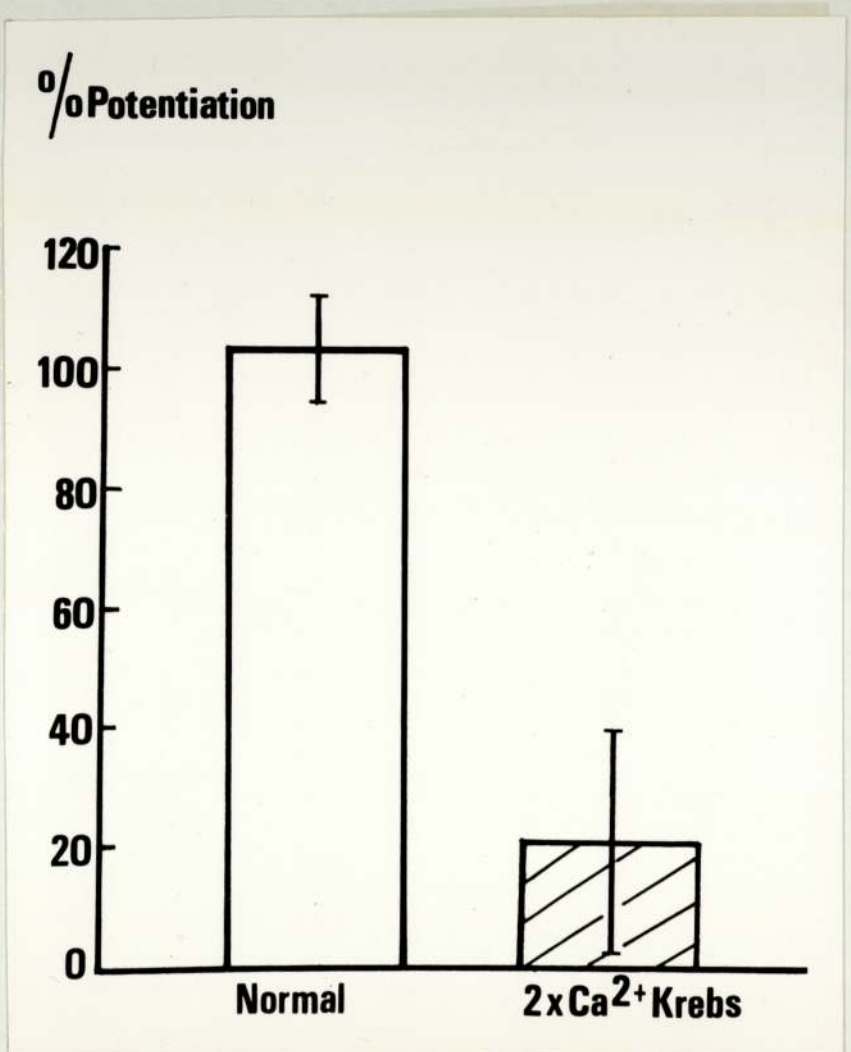

Fig. 38 Effect of incubating rabbit isolated aorta in Ca^{2+}-rich Krebs on the potentiation of NA (10^{-8} g/ml) by angiotensin II (10^{-9} g/ml). The open column is the % potentiation in normal Krebs, and the hatched column that in twice-normal Ca^{2+}-Krebs. N = 5.

by partially depolarising the membrane, hence making it more easily excitable, i.e. the threshold to other spasmogens would be lowered. An increased external potassium concentration would also be expected to have a similar effect, so the influence of an increased potassium concentration in the medium was examined.

Effect of raised extracellular potassium concentration on the potentiation of noradrenaline by angiotensin II

Doubling the concentration of potassium chloride in the medium resulted in a potentiation of the response to noradrenaline in each of five preparations. The results are summarised in Fig.39. The concentrations of noradrenaline had to be halved before angiotensin II was added to avoid an interference with angiotensin II's potentiating action. It can be seen that the peptide induced the same percentage potentiation in the presence and absence of raised potassium chloride, and this was true of three of the four preparations. In the remaining preparation, there was a 30% reduction in the size of the potentiation. Thus it appears that, in general, angiotensin II is not potentiating noradrenaline by a simple depolarising action, or by increased transmembrane movements of extracellular calcium.

DISCUSSION

Rabbit thoracic aortae contain endogenous stores of noradrenaline in the periarterial nerves (Maxwell et al, 1968) but do not contain endogenous stores of 5-HT (Maling, Fleisch & Saul, 1971). Aortae taken

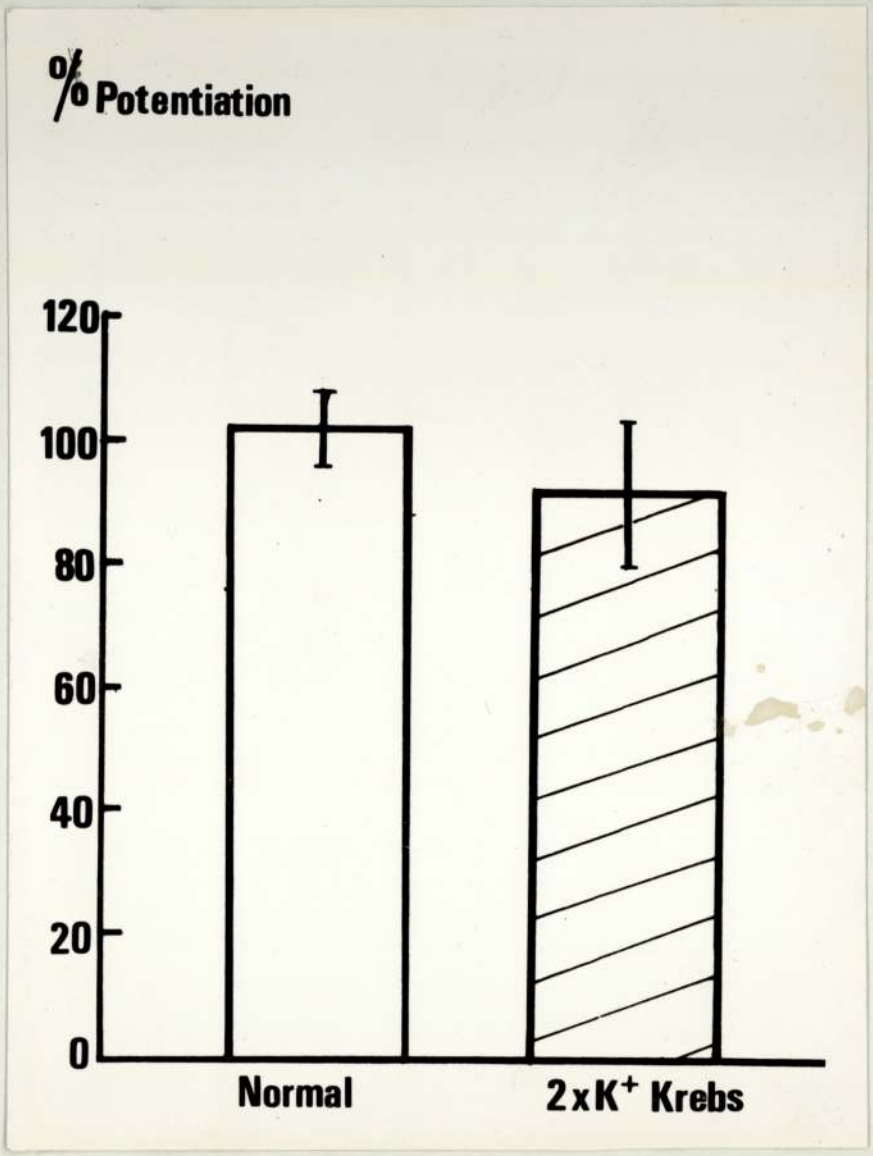

Fig. 39 Effect of incubating rabbit isolated aortae in K^+-rich Krebs on the potentiation of NA (10^{-8} g/ml) by angiotensin II (10^{-9} g/ml); open column is the % potentiation in normal Krebs, and the hatched column is that in 2 x K^+ Krebs (noradrenaline - 5 x 10^{-9} g/ml) N = 5.

from reserpinised rabbits have been reported to be supersensitive to noradrenaline, potassium chloride and acetylcholine, but not to angiotensin II, histamine or 5-HT. (Hudgins & Fleming, 1966).

Sakurai & Hashimoto (1965) observed a greater variation of the influence of reserpinisation on the responses of rabbit isolated vascular tissue to angiotensin II; they found that constrictor responses to angiotensin II were either unaffected or potentiated, when compared with controls. In the present study, this variability was repeated, in that two out of seven treated animals showed no increased reactivity to the constrictor response to angiotensin II whereas the other five did. This strongly suggests that the release of endogenous stores of noradrenaline is not involved in the direct constrictor action of angiotensin II on the rabbit aortae.

Khairallah, Page & Turker (1966b) found that indirectly-acting sympathomimetic amines potentiated the response to directly acting vascular spasmogens on rabbit aortae, and that this action was presumably due to release of endogenous noradrenaline as it did not occur in aortae taken from reserpinised rabbits. As it has been shown that angiotensin II can release noradrenaline from rabbit aortae (Kiran & Khairallah, 1969), albeit in high concentration (greater than 2.0×10^{-5} g/ml) it was essential to examine this

action as a possible mechanism for the potentiating action of angiotensin II. Reserpinised tissues exhibited the usual sensitivity to the potentiating action of angiotensin II, hence it is unlikely that a release of endogenous noradrenaline is involved in the mechanism of the potentiation.

The potentiation induced by angiotensin II is not specific to the sympathetic nervous system, as responses to potassium chloride and other agonists were potentiated to a similar extent as noradrenaline and were depressed by higher concentrations of angiotensin II; again as for noradrenaline. This strongly suggests a postsynaptic action of angiotensin II.

Garrett & Brown (1972) found that the potentiation of responses to 5-HT, noradrenaline and potassium chloride on the aorta by bradykinin (10^{-6} g/ml) was blocked in the presence of SKF 525A. Kalsner, Nickerson & Boyd (1970) had previously used this compound to specifically block responses to potassium chloride on the aortae, and as responses to potassium chloride are known to be due to an influx of calcium into the cell it seems reasonable to propose that SKF 525A has blocked this potassium-induced influx. In the present experiments, SKF 525A (10^{-5} g/ml) reduced the size of the contractile response to noradrenaline, blocked the response to potassium, but did not inhibit the potentiation of responses to

noradrenaline by angiotensin II. Therefore, if angiotensin II is acting by increasing influx of calcium, it is acting by a different mechanism to that of potassium chloride and bradykinin.

Various authors have suggested that non-specific increases in sensitivity to spasmogens of rabbit isolated aortae, induced by a variety of substances, are due to an increased calcium content of the tissue. This has been reported in the presence of ouabain (Briggs & Shibita, 1966), alcohol (Kalsner, 1970) bradykinin (Garrett & Brown, 1972) and tetraethyl-ammonium (Kalsner, 1973) and after pretreatment of rabbits with 6-hydroxydopamine (Shibita, Kuchi & Kurahashi, 1972) or reserpine (Carrier & Jurevics, 1972).

The direct spasmogenic action of angiotensin II has been shown to be dependent on the presence of extracellular calcium in the following tissues: frog isolated stomach muscle and heart (Singh, 1964), rat uterus and guinea pig ileum (Khairallah, Vadaparamil & Page, 1965), perfused mesenteric arteries (Burks, Whitacre & Long, 1967), and perfused rabbit kidney (Kline & Buckley, 1969).

Many reports have linked angiotensin II with a variation in tissue cation levels, and in the case of calcium, angiotensin II could act by increasing influx of calcium into the cell or by increasing release or decreasing rebinding of calcium from the cell membrane.

In the presence of high concentrations of angiotensin II, an increased uptake of calcium across the cell membrane has been reported in guinea pig isolated taenia coli (Shibita, Carrier & Frankenheim, 1968) and a shift of calcium from extracellular to intracellular sites in the rat isolated uterus (Khairallah, 1971). Angles d'Auriac, Baudouin & Meyer (1972) found in rabbit isolated aorta that angiotensin II in concentrations ranging from 10^{-9} to 10^{-7} g/ml caused an inhibition of calcium binding to the cell membrane at the lower concentrations, whilst at the higher concentrations it increased release of calcium from the cell membrane. The lowest concentration of angiotensin II (10^{-9} g/ml) shown by Angles d'Auriac et al (1972) to produce an effect on calcium binding corresponds with the concentration producing a maximum enhancement of noradrenaline in the present experiments. Their data correlate well with a calcium involvement in the contractile responses to angiotensin I^{I} rather than with its effect in sensitising the tissue to other spasmogens.

Unlike that induced by angiotensin II, the potentiation of noradrenaline by 5-HT is blocked in a calcium-free medium. However, 5-HT does potentiate the responses to spasmogens of aortae taken from reserpinised animals, so this block in a calcium-free medium is due to a post- rather than a pre-synaptic action. Thus 5-HT may be included in the list of compounds which appear to induce a non-specific

increased reactivity of rabbit isolated aortae via an increased intracellular calcium concentration.

EDTA has been reported to increase the response of isolated aortae to catecholamines by prevention of breakdown via heavy metal oxidation due to impurities in the organ bath (Shida, Meyers & Barker, 1963). In the present experiments, EDTA had a slight potentiating action on the response to noradrenaline, indicating some contamination of the bathing medium, but this did not prevent the angiotensin II induced potentiation of responses to noradrenaline. However, when the tissue was exposed to calcium free, EDTA-containing medium, the potentiation was abolished. There are at least two interpreations of this result :-

(1) The calcium "pool" which angiotensin II utilises to induce a sensitisation is different, and less easy to deplete, than that used by 5-HT.

(2) As Daniel (1965b) has observed, this treatment also inhibits the sodium pump, and it may be this action rather than the lack of extracellular calcium that is directly involved in the blockade of the potentiation.

The effect of excess calcium, a blockade of the potentiation, appeared to be a membrane-stabilising action, even though responses to noradrenaline were unaffected. It did not support the idea that

angiotensin II was increasing calcium influx, as one would expect an increased calcium influx with a greater external concentration of calcium. However, the lack of effect of depolarising the membrane with potassium chloride suggests that angiotensin II is not acting simply by depolarising the membrane and hence making the vascular smooth muscle cells of the aorta more responsive to drugs.

CHAPTER V

THE INVOLVEMENT OF SODIUM AND POTASSIUM IONS IN THE POTENTIATION OF NORADRENALINE BY ANGIOTENSIN II

INTRODUCTION

The results described in the previous chapter led to several questions being asked about the nature of the potentiation, the main one being whether or not the sodium pump was involved. The problem was approached initially indirectly, by the use of procedures known to inhibit energy-requiring processes, and the later approach was direct: to examine the influence of procedures known to block the sodium pump directly.

Skou (1965) reviewed in detail the characteristics of a membrane sodium pump, i.e. a sodium-potassium linked pump, stimulation of which enhanced the efflux of sodium ions from the cell and the influx of potassium ions into the cell. Recently, Thomas (1972) has reviewed the existing evidence as to whether or not this pump is electrogenic in nature, i.e. the exchange of sodium for potassium is not on a one for one basis, hence possibly giving rise to electrical disturbances of the membrane. The sodium pump of vascular smooth muscle is inhibited by ouabain or incubation in potassium-free medium (Garrahan, Villamil & Zadunaisky 1965), however, whereas ouabain prevented only the efflux of sodium from the cell, incubation in potassium-free medium prevented both efflux and influx of sodium ions.

Besides a sodium-potassium pump, several investigators have suggested the existence of a calcium-sodium pump on the vascular smooth muscle membrane (see Historical INTRODUCTION) Further discussion of this pump, and its possible role in the phenomenon presently being investigated will be delayed until the "Discussion" section of this chapter.

RESULTS

Effect of a Decreased Temperature

Lowering the temperature of the incubation medium to 20°C significantly decreased the size of the responses to noradrenaline, so that four times the control concentration of noradrenaline was used to produce similar-sized responses, the responses were also much slower than usual. However, as can be seen from the Summary of the results in Fig.40(a), there was no significant decrease in the percentage potentiation of noradrenaline by angiotensin II. However, this does not necessarily indicate that a temperature-sensitive process is not involved since a temperature of 4°C has to be reached before inhibition of the sodium-potassium pump is attained (Skou, 1965).

Effect of Oxygen -Lack

Four preparations were incubated in the usual way and control responses to noradrenaline were then obtained. The gas supply of the tissue was then removed, and aortae equilibrated in non-aerated

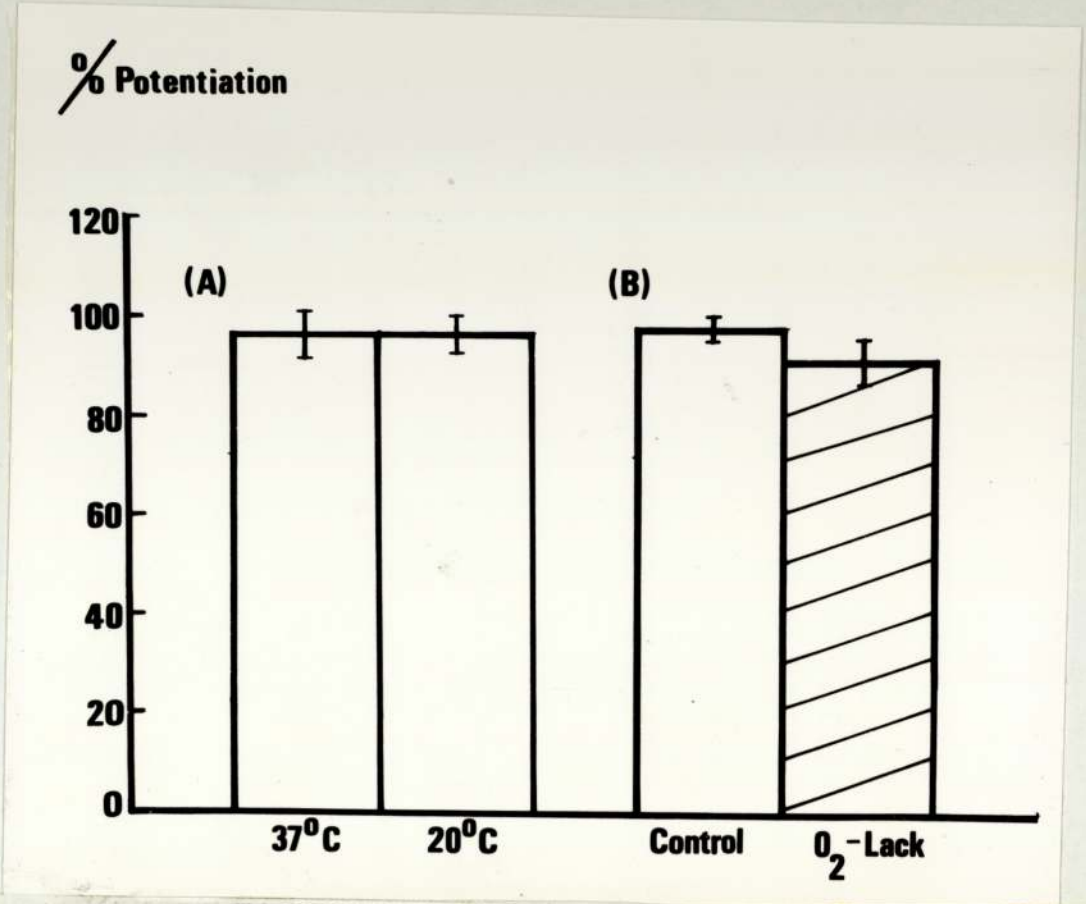

Fig. 40 Rabbit isolated aorta.
(A) Effect of lowering temperature on potentiation of NA by angiotensin II. Open column; noradrenaline (10^{-8} g/ml) potentiated by angiotensin II (10^{-9} g/ml) at 37°C. hatched column; NA (4×10^{-8} g/ml) potentiated by angiotensin (10^{-9} g/ml) at 20°C. N=4.
(B) Effect of O_2-lack on potentiation of NA by angiotensin II. Open column: NA (10^{-8} g/ml) potentiated by angiotensin II (10^{-9} g/ml); hatched column: NA ($10^{-8} \times 1.5$ g/ml) potentiated by angiotensin II (10^{-9} g/ml) in an O_2-deficient medium. N=4 for each point.

medium for 1 hr. At the end of this second incubation responses to noradrenaline were found to be smaller than control values (1.5×10^{-8} g/ml of noradrenaline producing responses approximately equal in size to those produced by 10^{-8} g/ml in control tissues. However, the potentiation of responses to noradrenaline by angiotensin II was unaffected by this treatment. The results of these experiments are summarised in Fig.40 (b).

Effect of Lack of Substrate

In this series of experiments, the usual carbohydrate substrate glucose was replaced by isosmolar concentrations of either sucrose, galactose or fructose. The results are summarised as a histogram in Fig.41.

a) Glucose replaced by Sucrose Replacement of glucose by sucrose had no significant effect on the size of the response to noradrenaline. However, it did prevent the angiotensin II-induced potentiation of responses to noradrenaline in each of four preparations.

b) Glucose replaced by Galactose Replacement of glucose by galactose had no significant effect on the response to noradrenaline in each of four preparations. Also, there was no significant effect on the percentage potentiation of responses to noradrenaline by angiotensin II.

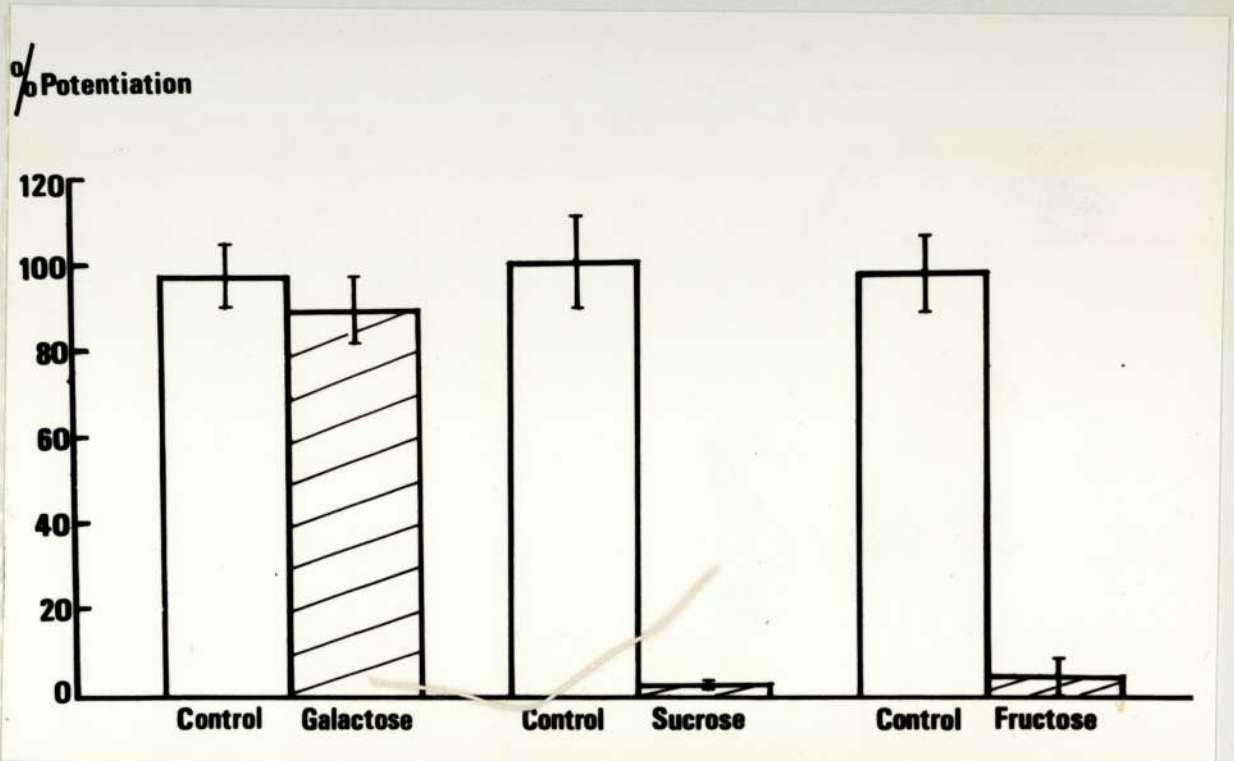

Fig. 41 Rabbit isolated aorta.

(A) Effect of replacing glucose by galactose on potentiation of NA (10^{-8}g/ml) by angiotensin II (10^{-9}g/ml) open column - glucose present, hatched column - glucose replaced by galactose. N=4.

(B) Effect of replacing glucose by sucrose on potentiation of NA (10^{-8}g/ml) by angiotensin II (10^{-9}g/ml) open column - glucose present, hatched column - glucose replaced by galactose. N=4.

(C) Effect of replacing glucose by fructose on potentiation of NA (10^{-8}g/ml) by angiotensin II (10^{-9}g/ml) open column - glucose present, closed column - glucose replaced by fructose. N=5.

c) Glucose replaced by Fructose Replacement of glucose by fructose had no significant effect on the response to noradrenaline in each of five preparations. However, the subsequent addition of angiotensin II (10^{-9} g/ml) produced no enhancement of the noradrenaline responses in four preparations, and a much smaller than normal potentiation in the remaining preparation.

These results suggest the involvement of a metabolism-linked active transport process, such as a membrane ion pump (Skou, 1965), in the potentiating action of angiotensin II. Replacement of glucose by either sucrose or fructose had no significant effect on the depression of responses to noradrenaline induced by constrictor concentrations of angiotensin II.

Effect of alteration of ionic composition of the bathing medium

a) Absence of Potassium In potassium-free bathing medium, responses to noradrenaline were slightly, but not significantly, reduced in each of five preparations. Contractions induced by angiotensin II (2.5×10^{-9} to 10^{-8} g/ml) were significantly reduced by this treatment. The potentiation of the responses to noradrenaline by angiotensin II (10^{-9} g/ml) was abolished during exposure of the tissue to potassium free bathing medium, as shown by the experiment illustrated in Fig.42. Since exposure to potassium-free medium is well known to induce blockade of the sodium pump (Garrahan et al, 1965; Thomas, 1972) then the sodium pump may be involved in the potentiating action of angiotensin II.

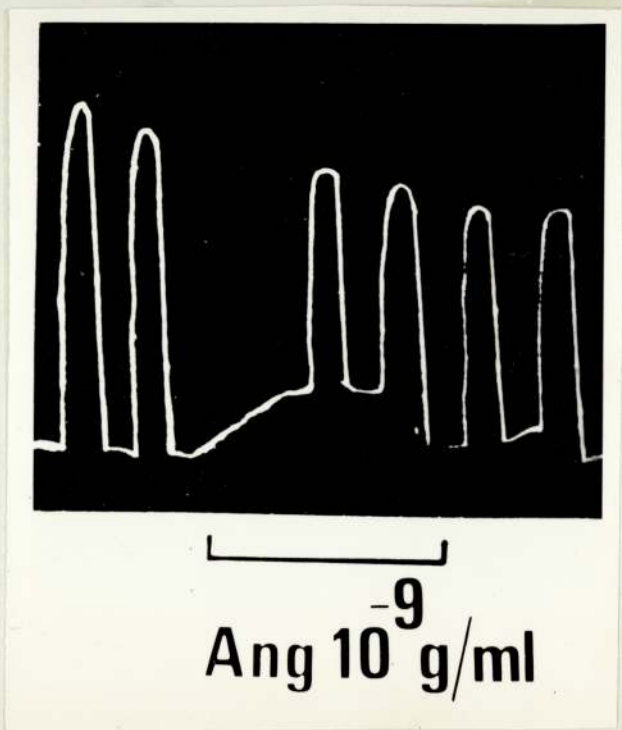

Fig. 42 Rabbit isolated aortic strip
Responses to noradrenaline (10^{-8} g/ml) were not potentiated by angiotensin II (10^{-9} g/ml) while the tissue was incubated in K^+-free Krebs solution.

Incubation of tissues in potassium-free medium had no significant effect on the depression of responses to noradrenaline induced by constrictor concentrations of angiotensin II.

b) Increase of Sodium A 20% increase in the sodium chloride content of the bathing medium increased the size of the responses to noradrenaline in all six preparations tested by approximately 100%, but had no significant effect on contractions to angiotensin II. In one experiment, the percentage potentiation of responses to noradrenaline by angiotensin II was significantly reduced on exposure of the tissue to sodium-rich bathing medium, in the other five experiments there was no significant difference between the percentage potentiation of noradrenaline by angiotensin II (10^{-9} g/ml) during exposure to normal or sodium rich bathing medium. Harris & Palmer (1972) increased the content of sodium by only 5% in the bathing fluid and found a significant increase in the $(Na)^{+}_{I}$: $(Na)^{+}_{O}$ ratio. Therefore, experiments were conducted using an increase of only 5% instead of 20% in the sodium chloride content of the bathing medium. A 5% increase in the sodium content of the bathing medium increased the size of the responses to noradrenaline in all four preparations tested by approximately 50%. Two concentrations of angiotensin II were applied to the tissue: 10^{-10} g/ml and 10^{-9} g/ml, it can be seen from the typical experiment illustrated in Fig.43 that the potentiation induced by the lower concentration of angiotensin II was inhibited in sodium-rich medium,

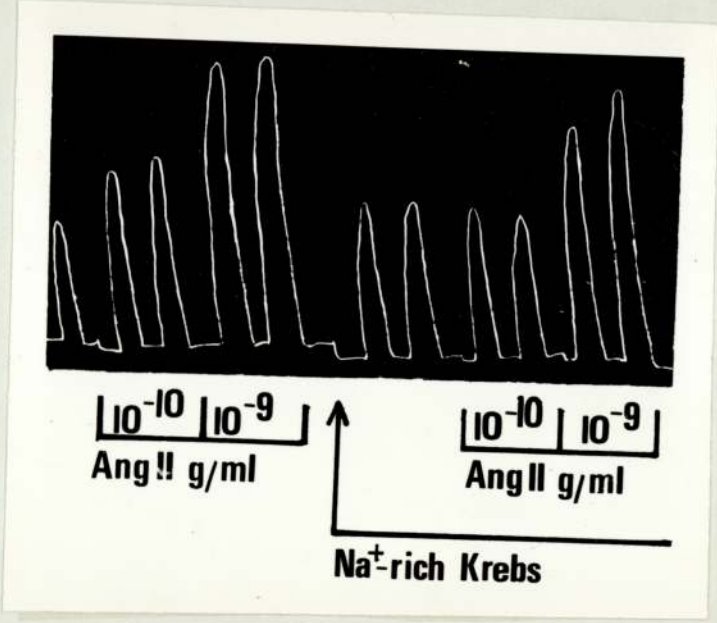

Fig. 43 Rabbit isolated aortic strip
Incubation in a Na^{+}-rich (5% increased) Krebs solution enhanced the responses to noradrenaline (10^{-8} g/ml) and reduced the angiotensin II-induced potentiation.

whereas that induced by the higher concentration of the peptide was not significantly affected.

c) Decrease of Sodium The sodium concentration of the medium was reduced by replacing half of the sodium chloride with an isosmolar quantity of sucrose. On contact of this solution with the tissue, a contraction ensued; the tissue relaxed to control base-line level over a period of twenty minutes. After an incubation period of one hour, the response to noradrenaline (10^{-8} g/ml) was significantly potentiated (over 100%) in comparison with controls. Subsequent additions of noradrenaline led, however, to a marked decline in the response, until the tissue becomes unresponsive to noradrenaline. This difficulty in obtaining steady responses to noradrenaline made it impossible to assess accurately the effect of angiotensin II on these responses.

Effect of Ouabain

Gulati & Jones (1971), using isotope-techniques found that ouabain at a concentration of 7.28×10^{-7} g/ml had a maximum blocking action on the sodium pump of the canine isolated carotid artery. Garrahan et al (1965) had previously observed that this concentration was maximally effective on the canine carotid. In the present series of experiments in each of six preparations, this concentration of ouabain increased the size of the submaximal contractions to noradrenaline by up to 100%, but prevented any further

enhancement in the presence of angiotensin II in a concentration (10^{-9} g/ml) which regularly increased the noradrenaline responses in control tissues. Fig.44 illustrates one out of this series of experiments; in the experiment illustrated, and in two others of this series, there was a contraction in the presence of angiotensin II (10^{-9} g/ml) although this concentration did not cause contractions in control tissues.

Effect of cocaine on ouabain - induced potentiation of responses to noradrenaline

In each of four preparations, exposure of the tissue to cocaine (4.5×10^{-5} g/ml) induced a significant (on average 100%) potentiation of the responses to noradrenaline. When the responses to noradrenaline had reached a steady level, addition of ouabain (7.28×10^{-7} g/ml) induced a further 100% potentiation above control values. A typical experiment is illustrated in Fig.45. Thus, although it has been demonstrated that ouabain does cause an inhibition of neuronal noradrenaline uptake in vascular smooth muscle (George & Leach, 1973), it is apparent that this is not the mechanism by which it is inducing a potentiation in the present experiments.

Effect of absence of extracellular calcium on ouabain-induced potentiation of noradrenaline

Exposure of arteries to calcium-free Krebs solution as has been noted in the previous chapter, reduced their responsiveness to noradrenaline by 75%. In

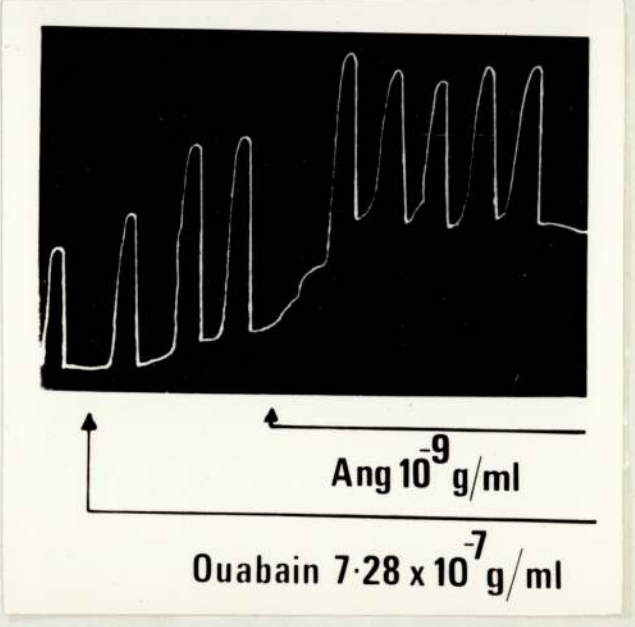

Fig. 44 Rabbit isolated aortic strip
Exposure to ouabain (7.28×10^{-7} g/ml) enhanced the size of the response to noradrenaline (10^{-8} g/ml) and addition of angiotensin II (10^{-9} g/ml) resulted in no further increase in size of the response to noradrenaline.

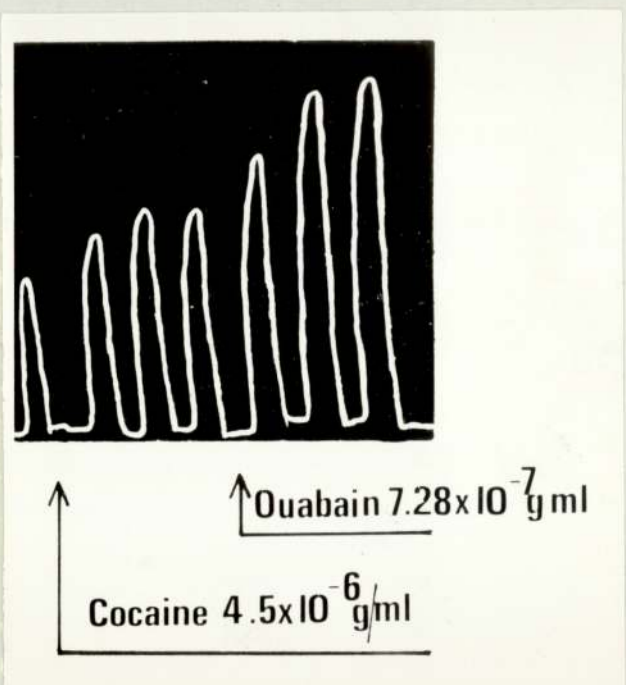

Fig. 45 Rabbit isolated aortic strip
Cocaine (4.5×10^{-5} g/ml) potentiated the size of the response to noradrenaline (10^{-8} g/ml). Addition of ouabain (7.28×10^{-7} g/ml) resulted in a further potentiation of the response to noradrenaline.

each of four preparations incubated in calcium-free medium, addition of ouabain (7.28×10^{-7} g/ml) had no potentiating action on the responses to noradrenaline, although in normal calcium containing medium this concentration of ouabain induced a 100% potentiation of noradrenaline responses. However, even though, in calcium-free medium, ouabain no longer induced a potentiation of responses to noradrenaline addition of angiotensin II (10^{-9} g/ml) still caused no enhancement of noradrenaline, as can be seen from the experiment illustrated in Fig.46. Therefore, although ouabain and angiotensin II appear to induce potentiations by different mechanisms, the potentiation induced by angiotensin II is still blocked by ouabain.

Effect of ethacrynic acid on the potentiation of noradrenaline by angiotensin II

Until comparatively recently, it was generally accepted that all active sodium transport was inhibited by cardiac glycosides. However, Hoffman & Kregenow (1966), using red blood cells, observed a component of active sodium efflux which was not inhibited by ouabain, but was inhibited by ethacrynic acid. They suggested that this component of sodium efflux was non-ATP dependent, and they called it "Pump II".

In the present experiments, four preparations were exposed to ethacrynic acid (3.03×10^{-6} g/ml) a concentration, although lower than that used by Hoffmann & Kregenow (1966) was equivalent when compared

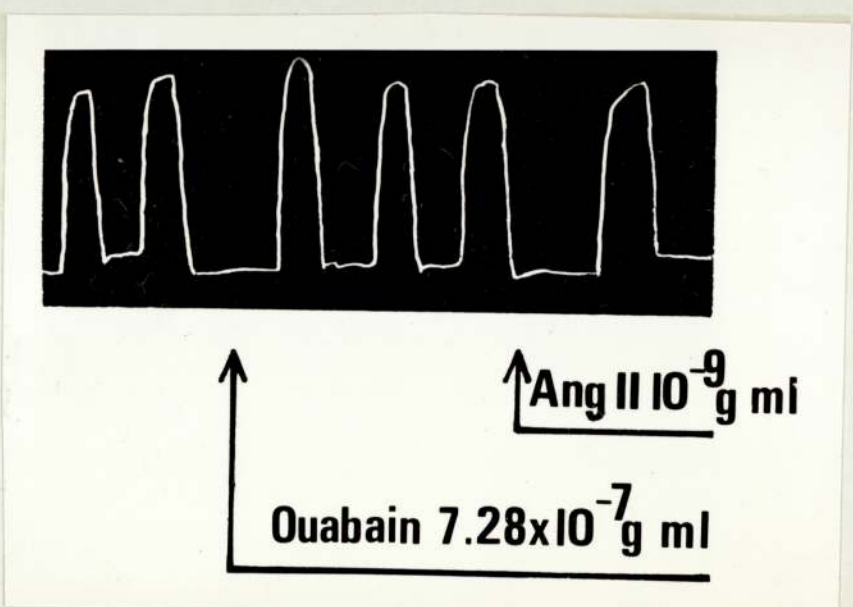

Fig. 46 Rabbit isolated aortic strip
Incubation in Ca^{2+}-free Krebs prevented the ouabain-induced potentiation of responses to noradrenaline (4×10^{-8} g/ml), and addition of angiotensin II (10^{-9} g/ml) in the presence of ouabain (7.28×10^{-7} g/ml) did not potentiate the response to noradrenaline.

with the relative concentration of ouabain, and had the advantage of having no action on membrane ATP-ase activity (Smith & Welt, 1973). It can be seen from the experiment illustrated in Fig.47 that ethacrynic acid had no significant effect on the responses to noradrenaline, nor did it have a significant effect on the percentage potentiation of noradrenaline by angiotensin II (10^{-9} g/ml).

DISCUSSION

The lack of effect of decreasing the temperature on the angiotensin II-induced potentiation of noradrenaline does not rule out a possible role of membrane ion pumps in the mechanism of the potentiation. The sodium pump is not totally inhibited until the temperature is lowered to 4°C (Skou, 1965), therefore, lowering the incubating mixture to room temperature (20°C) would have little effect on this process. However, there was a marked reduction in the sensitivity to noradrenaline at this temperature.

Furchgott (1966) found that provision of substrate (glucose) to anoxic rabbit aortae incubated in substrate-free medium for 1 hour enabled a 70% of normal contraction to adrenaline to occur; without the glucose there was no response. When gassed with oxygen, the tissue responded normally. In the present experiments, anoxia had no effect on the percentage potentiation of noradrenaline by angiotensin II, indicating that

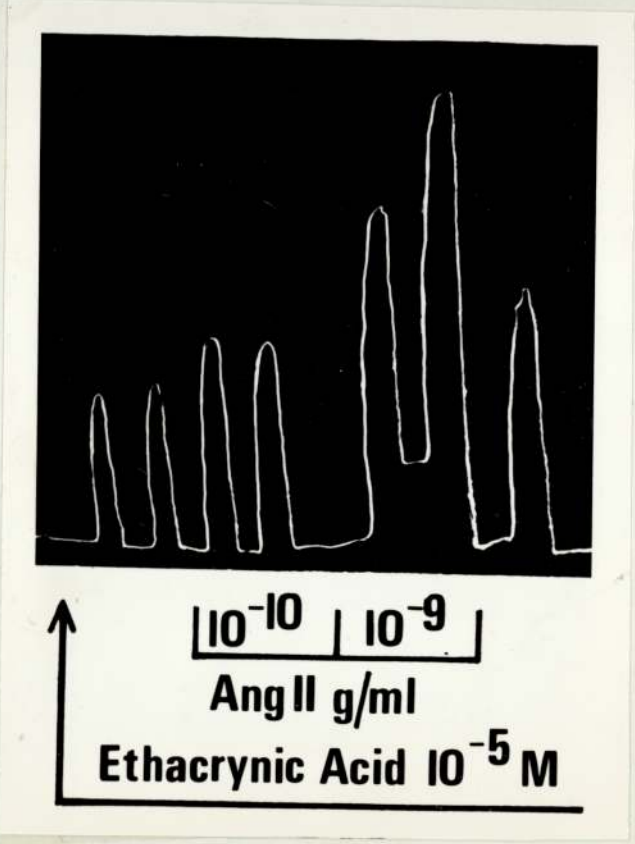

Fig. 47 Rabbit isolated aortic strip
Incubation in ethacrynic acid (10^{-5}M) did not inhibit the potentiation of responses to noradrenaline (10^{-8}g/ml) by angiotensin II (10^{-9}g/ml).

the energy required for this process is probably as equally available from glycolysis as from oxidative phosphorylation.

The substrate-dependent nature of contractions of rabbit isolated aorta observed by Furchgott (1966) were also reported by Coe, Detar & Bohr (1968). However, they used aerobic conditions and depleted substrate by continuous applications of adrenaline in substrate-free (i.e. glucose-free) medium. They found that, on addition of either glucose or mannose to the bath, responses of a depleted aorta to adrenaline would return, but fructose and xylose were ineffective in returning the responsiveness, suggesting a selective sugar transport. Earlier work by Wertheimer & Ben-Tor (1962), using rat aorta, supports this proposition, as they observed insulin-stimulated uptake of glucose, galactose, xylose and arabinose but no uptake of fructose.

The potentiation by angiotensin II of responses to noradrenaline was not blocked when the monosaccharide galactose was substituted for glucose, but was blocked when glucose was replaced by either the disaccharide sucrose or the monosaccharide fructose. From the study of Wertheimer & Ben-Tor (1962) it appears that galactose is a sugar which is transported in aortae, and apparently it can be used in the present case. However, sucrose, which is commonly

used as a neutral osmotic substitute for ions, and fructose, which apparently is a non-transported sugar in aortae (Wertheimer & Ben-Tor, 1962; Coe, Detar & Bohr, 1968) are incapable of replacing glucose in the role it plays in the potentiation of noradrenaline by angiotensin II.

The glucose-dependent nature of the angiotensin II-induced potentiation of responses to vascular spasmogens suggests the involvement of an active transport process. Bohr, Seidel & Sobieski (1969) presented evidence suggesting the existence of a sodium-calcium pump in the rabbit aorta, pumping sodium out of and calcium into the cell. However, the absence of a marked calcium dependency for the enhancement recorded in Chapter IV suggests that this pump is not involved; the observation that severe calcium depletion does inhibit the process may be further evidence that a sodium-potassium rather than a sodium-calcium pump is involved, as noted by Daniel (1965b).

Reports of an action of angiotensin II on sodium and potassium fluxes are variable. Friedman & Friedman (1964) found that constrictor concentrations of angiotensin II, in common with those of other vaso-constrictors, caused a shift of sodium ions into and potassium ions out of vascular smooth muscle cells. Daniel (1965a) found that large concentrations of angiotensin II (5×10^{-8} g/ml to 5×10^{-7} g/ml)

effectively inhibited reaccumulation of potassium by rabbit aortic strips recovering from sodium loading, but the sodium loss was unaffected. Rorive, Hagemeijer & Schoffeniels (1967) reported a transient increase in potassium efflux from perfused rat aortic preparations in response to angiotensin II, whilst Turker, Page & Khairallah (1967) found that submaximal concentrations of angiotensin II, but not 5-HT or bradykinin, increased sodium efflux from isolated canine carotid arteries and rat uterus. Khairallah (1971), however, felt that efflux was occurring during a period of repolarisation of the membrane, even though Turker et al (1967) observed a blockade of the angiotensin II-induced contraction of the uterus by ouabain. Szalay (1969) reported an inhibition of potassium accumulation in isolated pig adrenals during incubation with a very high concentration of angiotensin II (2×10^{-4} g/ml). In perfused rat caudal artery, Guignard & Friedman (1971) found that subconstrictor concentrations of angiotensin II caused a loss of potassium from the perfusing medium and a gain in sodium, whereas constrictor concentrations caused a smaller gain in sodium and a non-significant change in potassium levels.

Barbour, Gill & Bartter (1964) and Coviello & Crabbe (1965) found no significant action of angiotensin II in the concentration range 2.5×10^{-7} to 10^{-5} g/ml, on sodium transport in the toad skin and toad bladder; however, the authors note that angiotensin II was largely inactivated during contact with

the toad tissues.

In vivo, Villamil, Nachev & Kleeman (1970) observed that long-term (5-6 weeks) treatment of dogs with angiotensin II (via continuous intravenous infusion) resulted in an increased sodium content of the arterial walls.

Either incubating the tissue in potassium-free medium or exposing it to ouabain, both well-known sodium-pump blocking procedures, prevented the potentiation of responses to noradrenaline by angiotensin II. However, ouabain itself induced a potentiation of responses to noradrenaline, whereas potassium-free medium did not. The main difference between these two procedures is that exposure to potassium-free medium blocks both the influx and efflux of sodium, whereas ouabain blocks only the efflux; therefore, in potassium-free medium one would expect little alteration in the $(Na)^{+}_{I}$: $(Na)^{+}_{o}$ ratio, whereas exposure to ouabain would induce a marked increase in the $(Na)^{+}_{I}$: $(Na)^{+}_{o}$ ratio, a situation which, as has been noted in Section B of the HISTORICAL INTRODUCTION will increase the reactivity of the smooth muscle cell.

Ouabain has been shown to block uptake of catecholamines in vascular smooth muscle (George & Leach, 1973) but this mechanism is not apparent in the present experiments since a maximally effective neuronal-noradrenaline uptake blocking concentration of cocaine

does not prevent ouabain-induced potentiations. Ozawa & Katsuragi (1974) have recently observed a non-specific potentiating action of ouabain on the responses to spasmogens of the isolated guinea pig vas deferens. They concluded that ouabain was acting via a dual mechanism; presynaptic and postsynaptic. The potentiation induced by ouabain was dependent on the presence of extracellular calcium, suggesting that ouabain increases influx of calcium into smooth muscle cells, as originally proposed by Briggs & Shibita (1966). In terms of the sodium-calcium pump suggested by Bohr, Seidel & Sobieski (1969) this action of ouabain is easily explained: ouabain increases intracellular sodium, therefore the sodium-calcium pump is activated to remove this cellular sodium and hence the cell gains calcium.

Angiotensin II, however, does not appear to act by exactly the same mechanism, as although its action is blocked by ouabain, even in calcium-free medium, it does not exhibit a similar dependency on external calcium levels.

The experiments involving alteration of medium sodium levels are difficult to interpret. However, it does appear that an increase in tissue sodium levels increases the responsiveness of the tissue, and a prolonged decrease decreases the responsiveness.

The results from the use of ethacrynic acid suggests that the "Pump II" proposed by Hoffman & Kregenow (1966) plays no part in the potentiating action of angiotensin II.

CHAPTER VI

THE INFLUENCE OF SOME ANTAGONIST DRUGS, AND ALTERED ION-LEVEL MEDIUM, ON THE CONTRACTOR ACTION OF ANGIOTENSIN II ON VASCULAR AND VISCERAL SMOOTH MUSCLE

INTRODUCTION

A great deal of conflicting evidence has arisen as to whether the response of a variety of tissues to angiotensin II is of a direct or an indirect nature. In blood vessels, Liebau, Distler & Wolff (1965) found strong evidence to indicate that the response to angiotensin II was mainly indirect, whereas Khairallah et al (1966a) and Palaic & Lemorvan (1967) found that contractions of rabbit and guinea pig aortae, respectively, were direct in nature.

Suzuki & Matsumoto (1965) found that a range of anti-sympathetic drugs (including guanethidine, reserpine, cocaine and α-receptor blocking drugs) had a small (30%) but consistent reducing action on the spasmogenic response to angiotensin II using the rabbit isolated aortic strip. The results of other investigators cast doubt on the significance of these findings. Hong & Rodriguez (1965) found that reserpine in vitro had a non-specific depressant action on responses of rabbit isolated aortae, whereas Sakurai & Hashimoto (1965) found that vascular preparations taken from reserpinised rabbits were at least as sensitive to angiotensin II as control tissues.

Turker & Karahuseyinoglu (1968) reported a lack of effect of cocaine on angiotensin II-induced contractions of the rabbit isolated aorta, the concentrations of cocaine used (10^{-5} g/ml) totally blocking tyramine in the same preparation. They found that higher concentrations of cocaine reduced the action of angiotensin II, presumably via a local anaesthetic action. Shibita & Carrier (1967) reported a slight potentiation of responses to angiotensin II by α-receptor blocking drugs on the rabbit aortic strip; using high concentrations of phenoxybenzamine, dibenamine and chlorpromazine, they observed non-specific effects such as blockade of potassium-induced contractions, while those induced by angiotensin II were not significantly impaired.

The present chapter describes work undertaken to examine the specificity of the action of angiotensin II on the rabbit aorta, and the involvement of ions in this drug/receptor combination and transduction of the response. Also, contractor responses of the rat colon to angiotensin II were examined during alteration of intra:extracellular ion ratios.

RESULTS

Section A - Rabbit Isolated aorta

Effect of other spasmogens on the response to angiotensin II

(a) Noradrenaline Direct constrictor responses to angiotensin II (2.5×10^{-9} g/ml) were obtained on each

of four tissues. On exposure of these tissues to noradrenaline (10^{-9} g/ml) 2 min. before the following dose of angiotensin II, the response to angiotensin II was unaffected, as can be seen from the typical experiment illustrated in Fig.48. Concentrations of noradrenaline ranging from 0.75×10^{-9} g/ml to 2.0×10^{-8} g/ml had no significant effect on the size of the response to angiotensin II, whether they were added before any contact of angiotensin with the tissue, or between successive doses of angiotensin II.

(b) 5-HT and potassium chloride Sub-contractile concentrations of both 5-HT (10^{-8} g/ml) and potassium chloride (2.5×10^{-4} g/ml) induced a significant potentiation of the responses to angiotensin II (see Fig.49). As both of these compounds are known to release noradrenaline from sympathetic nerve endings the experiments were repeated using tissues taken from reserpinised animals. As can be seen from the results summarised in Fig.49, reserpinisation did not prevent either the potassium chloride or 5-HT-induced potentiation of contractile responses to angiotensin II.

Effect of some antagonist drugs on responses to angiotensin II

(a) Cocaine Cocaine at a concentration of 10^{-6} g/ml inhibited the response of the aorta to tyramine (10^{-6} g/ml) as can be seen in Fig.50(a). However, at the same concentration cocaine had no inhibitory action on the response to angiotensin II, Fig.50(b).

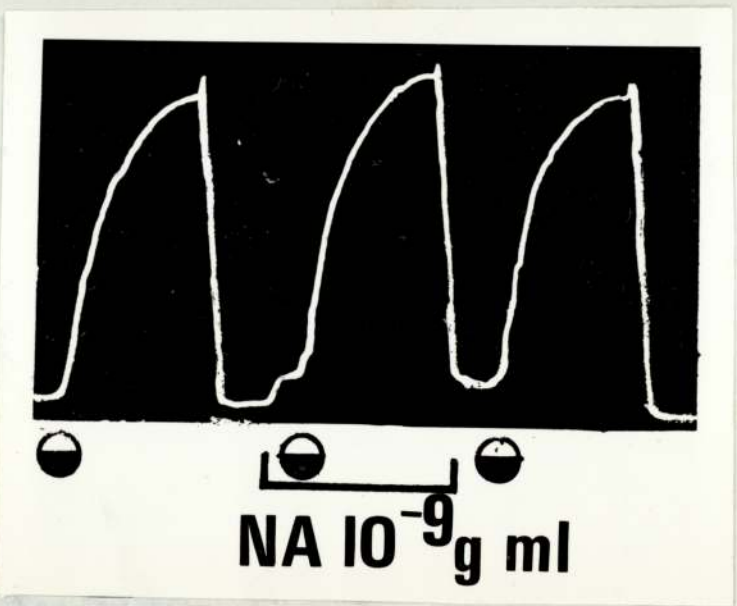

Fig. 48 Rabbit isolated aortic strip
Responses to angiotensin II (5×10^{-9}g/ml) were not altered in the presence of noradrenaline (10^{-9}g/ml).

Fig. 49

Agonist	Conc.^{n} g/ml	CONTROL % Pot^{n} of Ang.II	CONTROL SE±	CONTROL n	PRE-RESERPINISED % Pot^{n} of Ang.II	PRE-RESERPINISED SE±	PRE-RESERPINISED n
Noradrenaline	2.0×10^{-9}	0	0	6	-	-	-
KCl	2.5×10^{-4}	76.5	22.0	6	119.9	46.0	6
5-HT	10^{-8}	64.8	21.2	6	86.8	8.2	6

Fig. 49 Responses of the rabbit isolated aorta to angiotensin II (5×10^{-9}g/ml) were potentiated in the presence of either KCl (5×10^{-4}g/ml) or 5-HT (10^{-8}g/ml). Aorta taken from reserpinised rabbits also exhibited this potentiation.

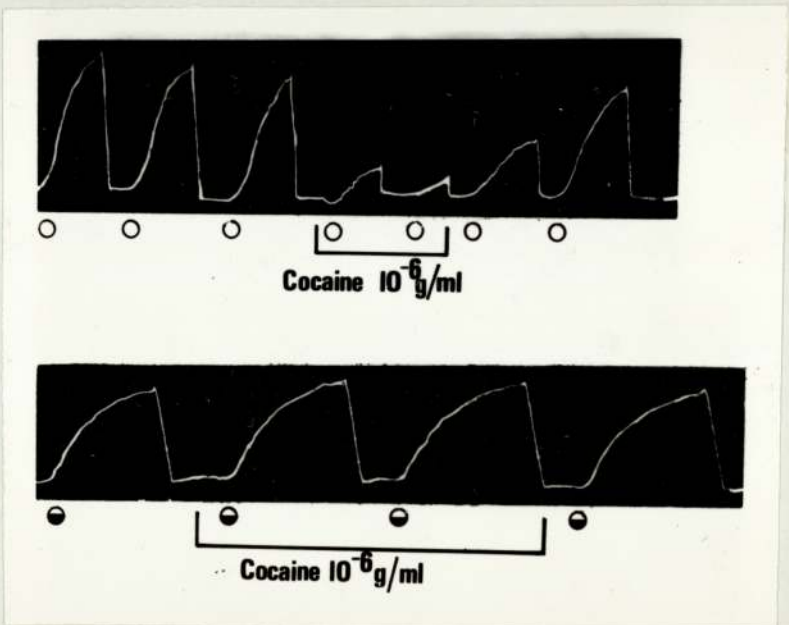

Fig. 50 Rabbit isolated aortic strip

(A) Responses to tyramine (○) (10^{-6}g/ml) were blocked by cocaine (10^{-6}g/ml) but recovered after it was washed out.

(B) Responses to angiotensin II (◒) (5 x 10^{-9}g/ml) were unaffected by cocaine (10^{-6}g/ml).

This indicates that angiotensin II was not acting on the sympathetic nerve via a cocaine-sensitive mechanism.

(b) Phentolamine At a concentration of 10^{-6} g/ml, phentolamine totally inhibited the response of the aorta to noradrenaline (see Fig.51(a)). However, at the same concentration, phentolamine had no significant effect on the responses to angiotensin II, indicating that angiotensin II was not inducing a response either by releasing noradrenaline from the nerve, or acting directly on the α-receptor itself. A typical experiment is illuatrated in Fig.51(b).

(c) Effect of pre-reserpinisation Tissues taken from reserpinised rabbits exhibited either an unchanged or an enhanced sensitivity to angiotensin II, again indicating a lack of involvement of neuronal noradrenaline in the response to angiotensin II. The results are summarised as a histogram in Fig.52.

(d) 5-HT antagonists Neither methysergide (10^{-6} g/ml) nor BOL-148 (10^{-6} g/ml) had an antagonistic action on the responses to angiotensin II, while responses to 5-HT were totally inhibited.

(e) P-113 P -113 (sarc^{1}, ala^{8}-angiotensin II) has been shown to block, selectively, both the direct constrictor action and indirect potentiating action of angiotensin II on vascular smooth muscle (Zimmerman, 1973). In the present experiments, in each of four preparations P-113 (10^{-8} g/ml) totally blocked the constrictor response to angiotensin II (5×10^{-9} g/ml).

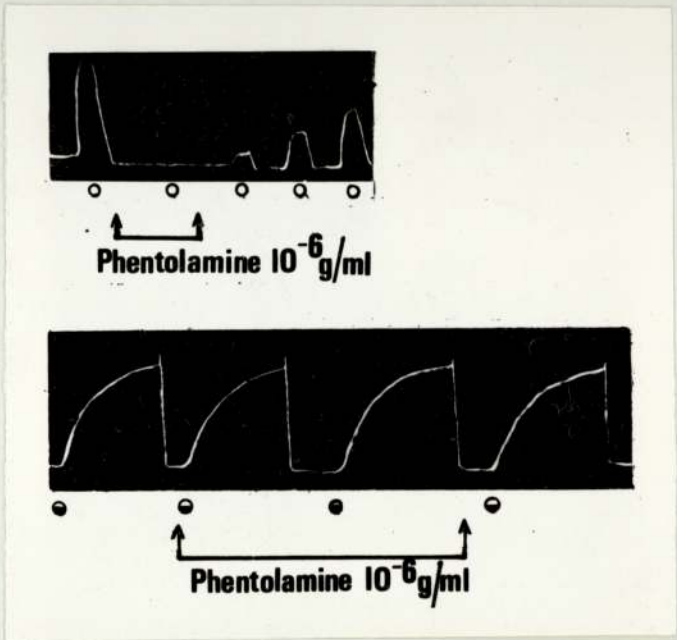

Fig. 51 Rabbit isolated aortic strip

(A) Responses to noradrenaline (10^{-8}g/ml) were blocked by phentolamine (2×10^{-6}g/ml) but recovered on removal of the drug.

(B) Responses to angiotensin II (5×10^{-9}g/ml) were unaffected by phentolamine (2×10^{-6} g/ml).

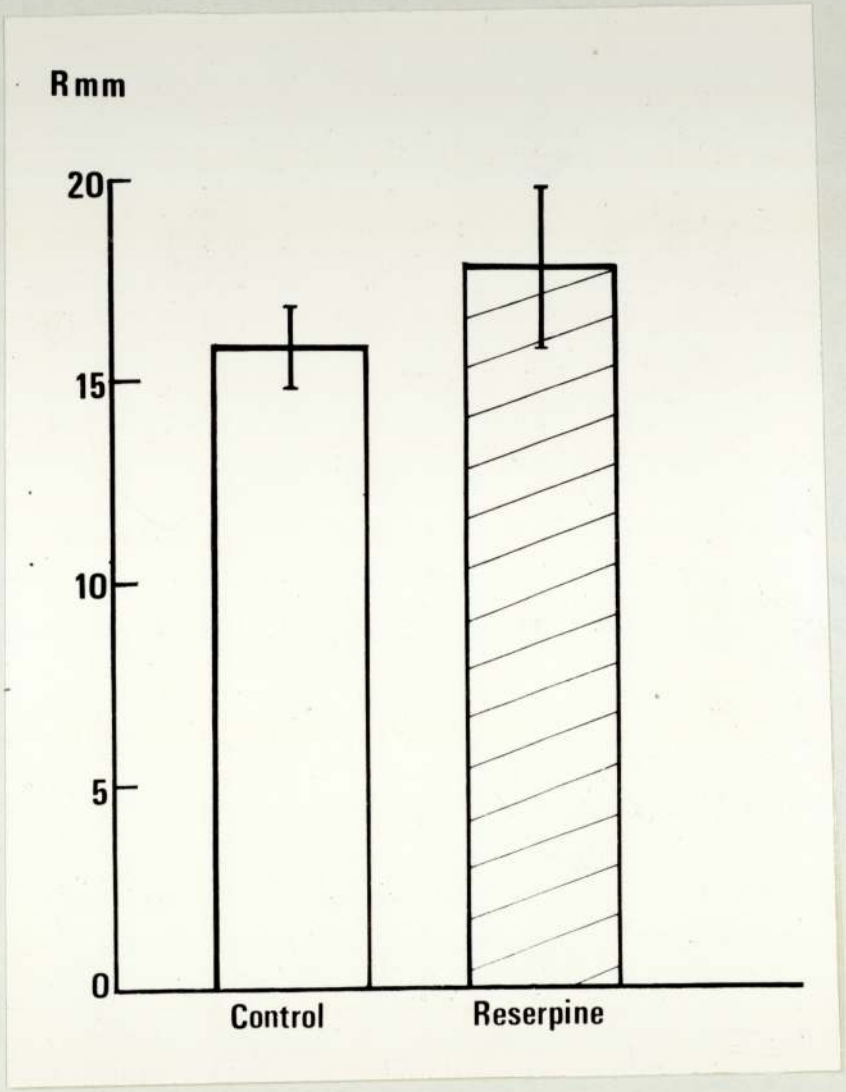

Fig. 52 Aortae taken from reserpinised rabbits exhibited either a normal or increased sensitivity to angiotensin II. The open column shows the response of normal aortae to angiotensin II (2.5 x 10^{-9}g/ml) and the hatched column shows the response of reserpinised aortae to angiotensin II (1.6 x 10^{-9}g/ml). N = 5 for each point.

A typical experiment is illustrated in Fig.53. It has been shown in previous experiments (see Chapter II) that P-113 had no inhibitory action on the responses of the aorta to noradrenaline. This, together with the results with phentolamine, suggests that angiotensin II and noradrenaline induce contractions of the rabbit isolated aorta by an action on separate receptors.

Tachyphylaxis

Goulet & Bohr (1959) found that responses of the rabbit isolated aorta to submaximal concentrations of angiotensin II were significantly reduced after 4 hours dosing of the tissue with angiotensin II using a 30 min. dose cycle, whereas responses to maximal concentrations were not affected, i.e. a refractoriness occurred at the lower end of the dose-response curve. Therefore, in the present experiments large concentrations (1.5×10^{-7} g/ml) of angiotensin II were used in an attempt to induce tachyphylaxis.

Tachyphylaxis to angiotensin II was difficult to obtain, an observation previously made by Khairallah et al (1966a). One out of the four tissues used still gave an unaltered response to angiotensin II after an 8 hour experimental period.

On attaining tachyphylaxis, tissues were incubated with noradrenaline (4.0×10^{-6} g/ml) for 30 min. and allowed to recover for 15 min. This

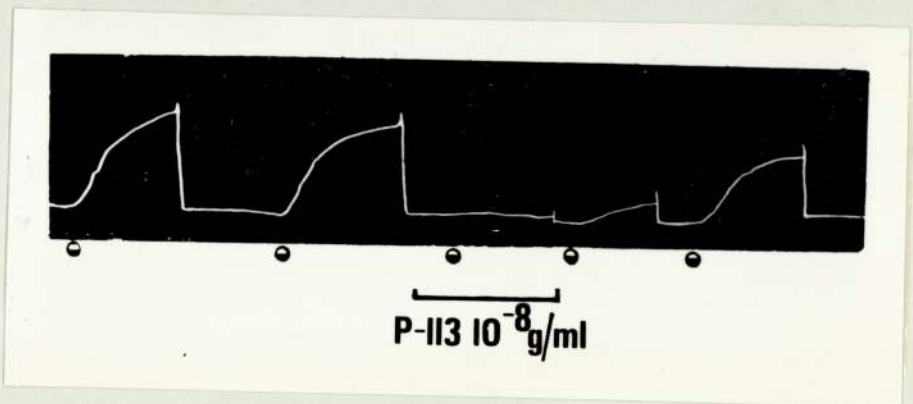

Fig. 53 Rabbit isolated aortic strip
Responses to angiotensin II (5×10^{-9}g/ml) were abolished in the presence of P-113 (10^{-8}g/ml). On washout of P-113, the responses to angiotensin II slowly returned to normal.

procedure reversed tachyphylaxis to tyramine (see Chapter III), but it did not reverse tachyphylaxis to angiotensin II, as can be seen from the experiment illustrated in Fig. 54.

Altered ion levels

(a) Absence of potassium Incubation of six preparations in K^+-free medium for 2 hours significantly reduced the response to angiotensin II without significantly altering that to noradrenaline. The results are expressed as a histogram in Fig. 55. Thus it appears that, like the angiotensin II-induced potentiation, the actual contraction induced by angiotensin II is mediated via an action on the sodium pump.

(b) Ouabain Ouabain, at a concentration of 7.28×10^{-7} g/ml, slightly potentiated responses to angiotensin II on the rabbit isolated aorta; it has previously been found that ouabain also potentiates responses to noradrenaline (see Chapter V). A typical experiment is illustrated in Fig. 56. The non-specific nature of the potentiating action of ouabain has recently been noted in other tissues, particularly the guinea pig vas deferens (Ozawa & Katsuragi, 1974).
In view of the results of incubation in a K^+-free medium, it appeared that angiotensin II was inducing a contraction via a direct action on the sodium pump. However, as ouabain potentiates and does not block the action of angiotensin II, then the process is apparently more complex.

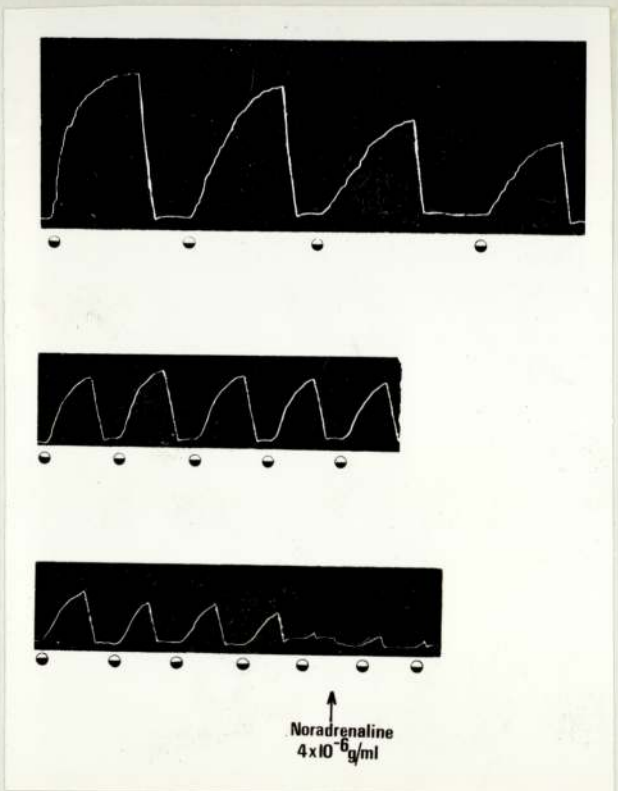

Fig. 54 Rabbit isolated aortic strip

Tachyphylaxis to angiotensin II (1.5×10^{-7} g/ml) was induced over a prolonged period. Incubation with noradrenaline (4×10^{-6} g/ml) for 30 min. did not reverse the tachyphylaxis.

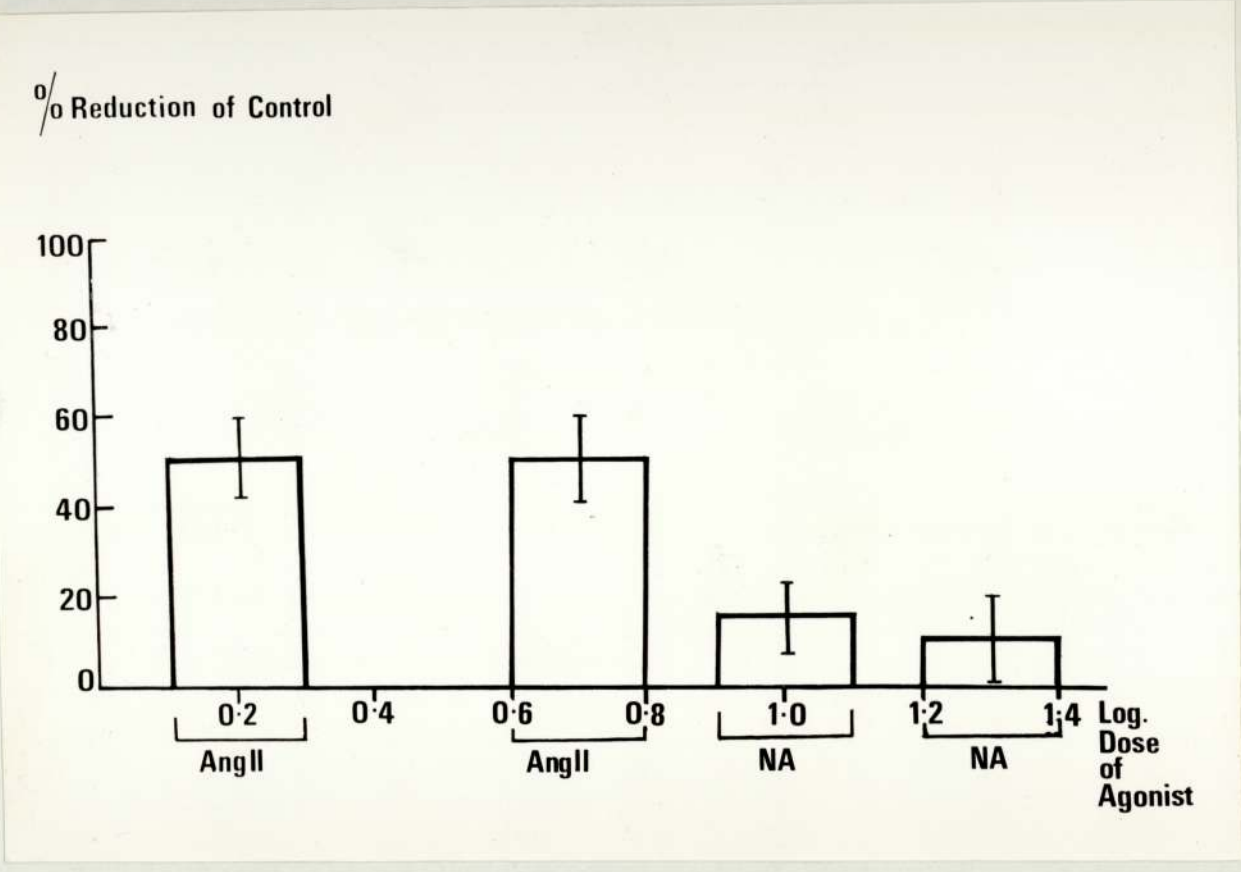

Fig. 55 Incubation of rabbit isolated aortae in K^+-free Krebs resulted in a significant reduction in the responses to angiotensin II (left-hand columns) but no significant reduction in the responses to noradrenaline (right-hand columns). N = 7 for each point.

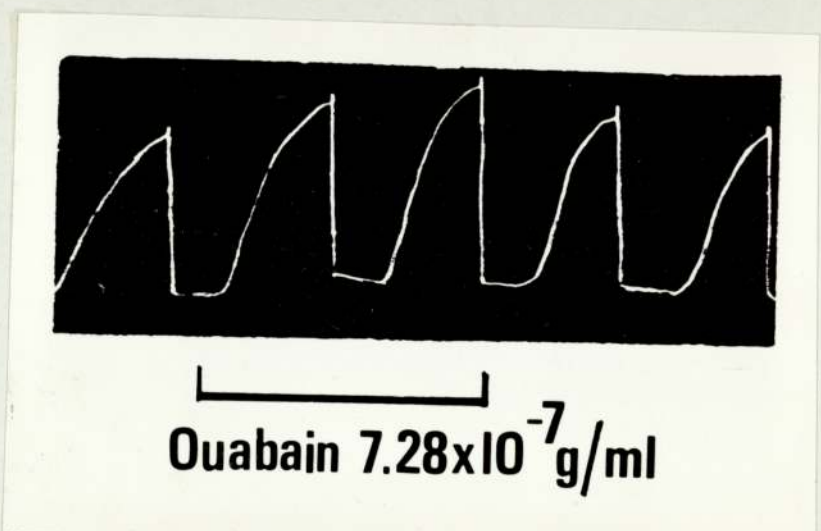

Fig. 56 Responses to angiotensin II (5×10^{-9}g/ml) were not significantly affected in the presence of ouabain (7.28×10^{-7}g/ml).

(c) Calcium and EGTA Lack of external calcium significantly inhibited responses to the aorta to angiotensin II. Concentrations of 2.5×10^{-9} g/ml to 10^{-8} g/ml of angiotensin II, which normally elicited a response, had no effect in a calcium-free medium. Responses to noradrenaline were also significantly reduced under these conditions, as has been observed in Chapter IV.

EGTA (10^{-5} g/ml), a calcium-chelating compound, reduced responses to angiotensin II and noradrenaline to a similar extent, i.e. it has a non-specific blocking action on the reactivity of the aorta.

Section B - Rat Colon

Responses of the rat colon to angiotensin II have been reported to be totally direct (Regoli & Vane, 1964), i.e. they do not involve release of neuronal acetylcholine, or any other action on the nervous elements.

(i) Ouabain At concentrations of 7.28×10^{-6} g/ml or below, ouabain had no significant effect on the resting tone or responsiveness of the tissue. At 7.28×10^{-5} g/ml, the basal tone of the tissue was raised, but the size of the spontaneous contractions was diminished in all six tissues tested; responses to both acetylcholine and angiotensin II were either reduced (four tissues) or not significantly affected (two tissues).

(ii) Potassium-free medium In each of four tissues a one hour exposure of the tissue to potassium-free Krebs had similar effects; the results of a typical experiment are illustrated in Fig.57. There was no alteration in basal tone of the tissue, but spontaneous contractions ceased; these contractions recurred on exposure to a normal medium. Responses to both acetylcholine and angiotensin II were initially unaffected, but on prolonged exposure to potassium-free medium, they declined significantly. Responses returned to control values on re-exposure to normal medium and the spontaneous contractions resumed. It appears that procedures inhibiting sodium fluxes have a non-specific depressant effect on visceral smooth muscle, particularly on the spontaneous contractions.

DISCUSSION

A possible explanation for the failure of other investigators to repeat the observations of Liebau et al (1965), that incubation with noradrenaline reverses angiotensin II-induced tachyphylaxis is that noradrenaline may be potentiating any remaining response to angiotensin II. This has been shown, in the present experiments, not to be the case. However, responses to angiotensin II were potentiated by both 5-HT and potassium chloride, and as this potentiation also occurred in aortae taken from reserpinised animals it was not due to release of neuronally-bound noradrenaline, i.e. both 5-HT and potassium chloride were acting post-synaptically.

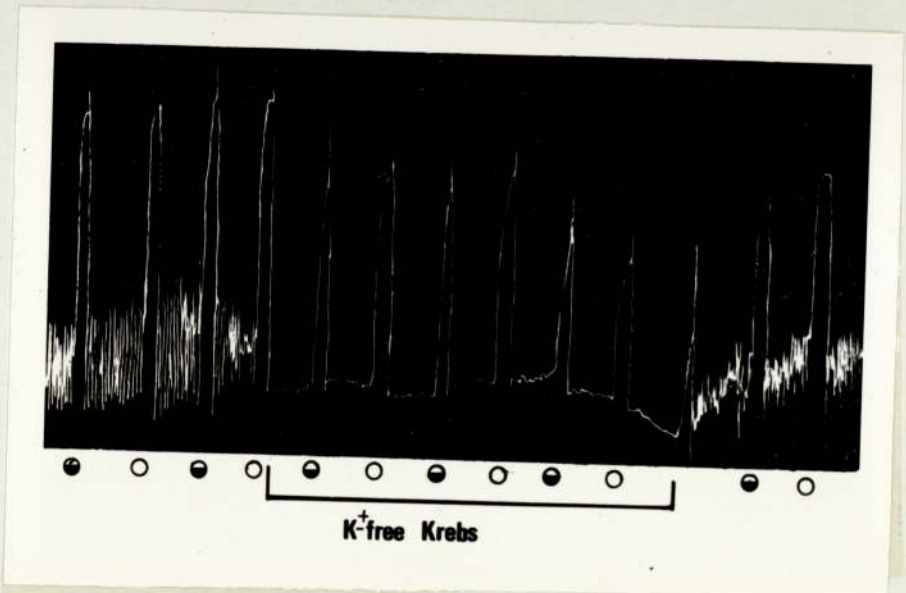

Fig. 57 Rat isolated colon

Responses to acetyl choline (10^{-7}g/ml) and angiotensin II (2×10^{-9}g/ml) were both reduced after prolonged incubation of the tissue in K^+-free medium.

Sponteneous motility of the tissue was also blocked.

Although cocaine has previously been reported to have an inhibitory action on the responses to angiotensin II both in rabbit aortae (Suzuki & Matsumoto, 1965) and in guinea pig aortae (Schumann & Guther, 1967), Turker & Karahuseyinoglu (1968) found no inhibitory action of cocaine on the responses to angiotensin II of the rabbit isolated aorta. This lack of effect of cocaine has been repeated in the present experiments using cocaine at a concentration which has been shown to block tyramine-induced contractions.

Angiotensin II induces a contraction of the rabbit isolated aorta via an action on its own receptors, rather than via 5-HT or α-adrenergic receptors; its action was unaffected by phentolamine, methysergide or BOL-148 in concentrations sufficient to block responses to noradrenaline and 5-HT respectively. Similar observations have recently been reported by Rioux, Park & Regoli (1973).

Tachyphylaxis to angiotensin II was difficult to induce on the rabbit aorta, and once induced was not reversed by incubation with noradrenaline. These findings agree with those of Khairallah et al (1966a). It appears, therefore, that in this tissue the action of angiotensin II is totally direct, an observation strengthened by the observations of Meyer & Baudouin (1971) who found that the degree of contraction

produced by angiotensin II in vitro on the rabbit aortic strip was the same in the presence or absence of the adventitia, i.e. in the presence of absence of sympathetic nerves (Bevan & Verity, 1967). Baudouin, Meyer & Worcel (1971) reported specific labelled angiotensin II binding sites on adventitia-stripped rabbit aortae, suggesting the presence of specific angiotensin II-receptors on the smooth muscle cell. The specific angiotensin II blocking action of P-113 further suggests the existence of specific angiotensin II receptors on the smooth muscle cells of rabbit aorta.

The variable nature of the results of studies of the effect of angiotensin II on sodium and potassium fluxes in smooth muscle has been described in detail in the "HISTORICAL INTRODUCTION". Meyer & Baudouin (1971) suggested that by supressing the activity of the pump which provides for the extrusion of sodium ions and the influx of potassium ions, angiotensin II depolarises the vascular smooth muscle cell membrane, with resultant passive enhancement of ionic permeability and increase in intracellular sodium ion. However, depolarisation is not an essential result of peptide-receptor interaction in the induction of a contraction, as the relative magnitude of the vascular smooth muscle response to angiotensin II is similar in the polarised and depolarised state (Somlyo & Somlyo, 1968). In the present experiments, ouabain potentiated the response to angiotensin II, whereas

incubation in potassium-free medium significantly reduced it. Both procedures are known to inhibit the efflux of sodium from the cells, whereas only incubation in potassium-free medium inhibits the influx of sodium into the cell (Garrahan et al, 1965). Thus it appears that there is a direct relationship between intracellular sodium levels and the reactivity to angiotensin II.

The responses of the rat colon to angiotensin II appear to be of a direct rather than an indirect nature (Regoli & Vane, 1964). Therefore, similar procedures were applied to this tissue to examine the possibility that influx of sodium ions into the smooth muscle cell was a general requirement for the action of angiotensin II, not just specifically for vascular smooth muscle. Inhibition of the sodium pump in visceral smooth muscle reduces the tissue sensitivity non-specifically. The most significant result of this series of experiments appears to be the effects on the spontaneous contractions of the colon. These were totally blocked on exposure of the tissue to potassium-free medium, during a period when the tissue was responding normally to acetylcholine and angiotensin II, presumably, this is due to a disturbance of the electrical events at the membrane.

C H A P T E R VII

THE EFFECT OF FRUSEMIDE ON THE CONSTRICTOR ACTION OF ANGIOTENSIN II AND OTHER SPASMOGENS ON THE PITHED RAT BLOOD PRESSURE

INTRODUCTION

Diuretics have been used as anti-hypertensive agents, either alone or in combination, for several years (Earley & Orloff, 1964; Conway, 1965; Hutcheon, 1967). They have variously been described as non-specific blocking agents of vascular spasmogens (Bock & Gross, 1960; Gillenwater et al, 1962; Blair-West et al, 1972) or ineffective as blocking agents (Silah et al, 1965; Napodano et al, 1962).

Recently, Perez-Olea, Quevedo, Munoz & Illanes (1969) found that treatment with frusemide significantly reduced the response to angiotensin II in pithed rabbits, while not affecting the response to tyramine and noradrenaline. This led to an examination, in the present chapter, of a possible angiotensin II-blocking action of frusemide on the blood pressure of the pithed rat.

RESULTS

Pretreatment with frusemide

Pretreatment with frusemide was carried out for periods varying from 3 hrs. before pithing (a single intra-peritoneal dose of 20 mg/kg) to daily treatment

at 10 mg/kg intra-peritoneally for seven days. On pithing, there was no significant difference in the response to nerve stimulation, noradrenaline and angiotensin II between treated and control rats.

Acute intra-peritoneal injection

Frusemide injected intra-peritoneally after controls to noradrenaline, angiotensin II and nerve stimulation had been obtained had no effect in each of six rats undergoing this procedure. The results of a typical experiment are illustrated in Fig.58. Increasing the concentration of frusemide to 20 mg/kg still had no significant effect on the responses to pressor substances.

Acute intravenous injection

In 17 out of 26 experiments, the response to angiotensin II was depressed by more than 50%, 2 to 3 hours after the injection of frusemide (10 mg/kg). A typical experiment is illustrated in Fig.59. In this series of experiments, there was slight, but not significant, reduction of the responses to nerve stimulation and noradrenaline.

In the other nine experiments, where frusemide had no significant effect on constrictor responses to angiotensin II, responses to noradrenaline and nerve stimulation were also unaffected. However, addition of a further 10 mg/kg of frusemide had a non-specific depressant effect, as can be seen in the typical example of this group of experiments illustrated in Fig.60.

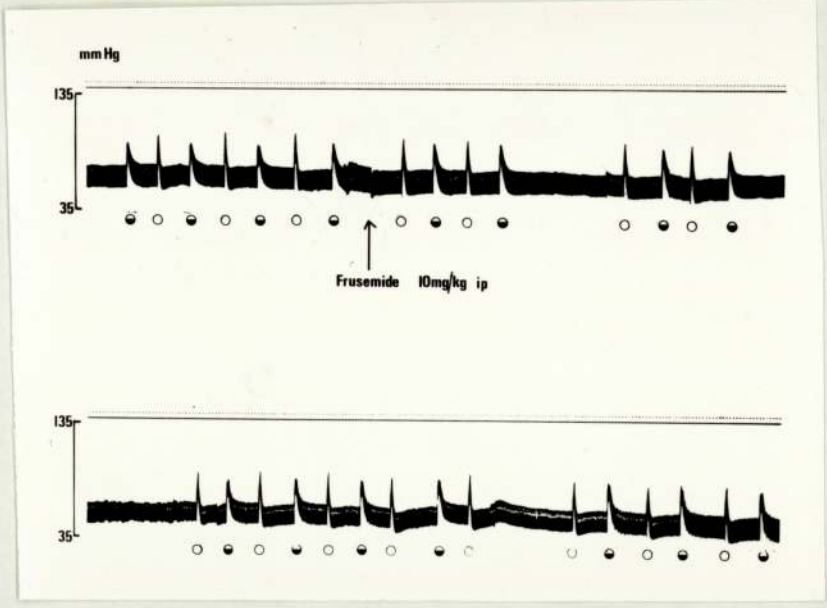

Fig. 58 Frusemide, 10mg/kg i.p. had no significant effect on the responses of the pithed rat blood pressure to either nerve stimulation (0.25Hz) or angiotensin II (5.0×10^{-8} g/kg).

nerve stimulation - o

and angiotensin II - ●

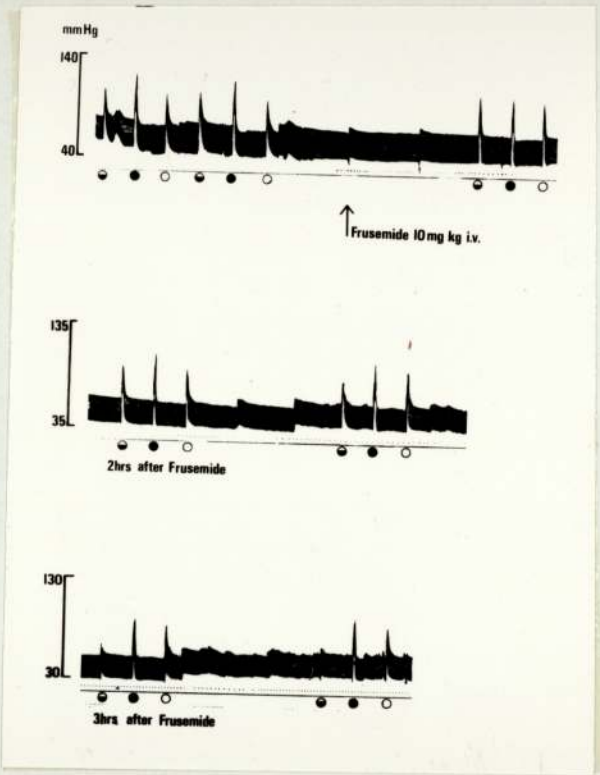

Fig. 59 Pithed rat blood pressure

Frusemide, 10mg/kg intravenous had, in this preparation, a significant blocking action on the response to angiotensin II (5.0×10^{-8} g/kg) while responses to noradrenaline (10^{-7} g/kg) and nerve stimulation (0.25Hz) were relatively unaffected.

Noradrenaline - - o
nerve stimulation - ●
and angiotensin II - ◒

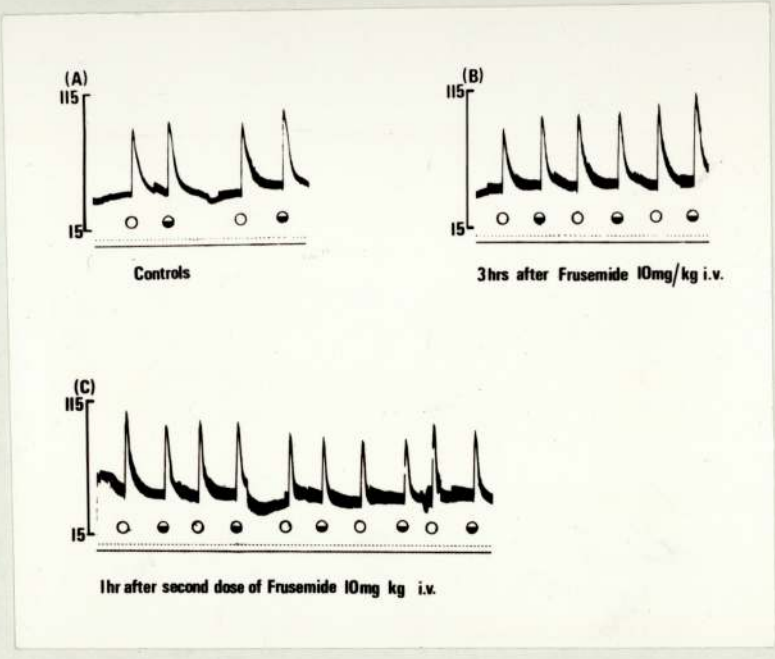

Fig. 60 Pithed rat blood pressure

In this preparation, frusemide 10mg/kg intravenous had no significant effect on the response to either angiotensin II (5.0×10^{-8}g/kg) or nerve stimulation (10^{-7}g/ml). A further injection of frusemide, 10mg/kg intravenous induced a depression of responses to both agonists.

nerve stimulation - O

and angiotensin II - ●

DISCUSSION

Frusemide does not have a clear-cut effect on the responses of the pithed rat blood pressure to vascular spasmogens. Finch (1971) found that treatment of hypertensive rats orally with frusemide reduced their hypertension to normotensive levels, while not affecting responses to nerve stimulation or noradrenaline in the pithed animals. The latter results have been repeated in the present experiments, although responses to angiotensin II were also unaffected. Acute intra-peritoneal injection of frusemide was also ineffective.

In just over half of the preparations tested, intravenous frusemide had a specific anti-angiotensin II effect, agreeing with the results of Perez-Olea et al (1969). However, in the other half, frusemide had little effect and in concentrations greater than 10 mg/kg had a non-specific depressant effect. These latter results are similar to those observed by Blair-West et al (1972) who found that frusemide had a non-specific depressant action on the responses of the rat isolated portal vein to a variety of vascular spasmogens, including noradrenaline and angiotensin II. It may be that there are "frusemide-sensitive" and "frusemide-insensitive" animals.

GENERAL DISCUSSION

GENERAL DISCUSSION

Although Bright (1836) first noted an association of kidney disease with elevated peripheral arterial pressure, it was not until the studies of Goldblatt, Lynch & Summerville, Hanzal (1934) and Page (1939) that this link was verified experimentally. These workers applied, respectively, occlusive renal artery techniques and kidney wrapping to whole animals and observed the resulting severe hypertension. Selye & Stone (1946) demonstrated that aortic constriction above the left renal artery in the rat, leaving an intact circulation to the right kidney, could produce a severe, nephrotic hypertension, even when the left kidney had lost its functional capacity to secrete urine. The continuation of the hypertensive process in the face of no excretory function led Selye to use the term "endocrine kidney".

These observations led to the study of the involvement of renin and angiotensin II in the development and maintenance of various forms of hypertension (recently reviewed by Capelli, Wesson & Housel, 1973). The role of angiotensin II is more likely to be of an indirect nature, rather than a direct contractor effect on smooth muscle cells, as the measured plasma levels of angiotensin II in hypertensive patients are not necessarily different from those of normotensive individuals (Catt, 1969). Recently, however, Crane, Genest & Sambhi (1973) have reported higher angiotensin II-generation rates on addition of homologous

renin to plasma, in vitro, from essential hypertensive patients in comparison with controls.

The first part of this thesis was involved with an investigation of the effect of angiotensin II on the responses to endogenous and exogenous noradrenaline in vascular smooth muscle preparations taken from the rabbit, as an attempt to elucidate an in vitro interaction of angiotensin II with the sympathetic nervous system. An interaction at this level may be involved in the genesis of some forms of hypertension, as Ayitey-Smith & Varma (1970) found that neither DOCA/saline hypertension nor sustained renal hypertension could be produced in "total" immuno-sympathectomised rats.

The sensitivity of the rabbit isolated ear artery to the constrictor action of angiotensin II was less than that of the isolated whole ear, an observation previously made by de la Lande & Rand (1965), who suggested that this was due to the absence of arterioles in the former preparation. This explanation is feasible, as Mellander & Johansson (1968) found that the main site of constrictor action of the polypeptide on the blood pressure of the whole animal was at the level of the arterioles.

At the lower values of infusion, angiotensin II had a potentiating action on responses to nerve stimulation, but no action on noradrenaline in the

isolated rabbit ear artery; higher concentrations induced a depression. On exposure of the outer surface of the artery to the polypeptide, there was a specific depression of responses to nerve stimulation at the lower concentration of angiotensin II, and a non-specific depressant effect at the higher concentration. It may be concluded that angiotensin II does reach the sympathetic nerve ending, and that in general, this effect is depressant in nature in this preparation. However, in both the rabbit isolated whole ear and rabbit isolated mesenteric bed, angiotensin II induced, in general, a potentiation of the response to nerve stimulation while having little or no effect on the response to noradrenaline. This picture of a potentiation of responses to endogenous but not exogenous noradrenaline has previously been observed during recording of the blood pressure of the anaesthetised dog by McCubbin & Page (1963), and also on the blood pressure of the pithed rat by Day & Owen (1969). Both groups of workers ascribed this action of angiotensin II to a facilitation of the release of noradrenaline from sympathetic nerve endings during nerve stimulation.

Other workers have also observed that angiotensin II potentiates endogenous rather than exogenous noradrenaline, such as in the rat isolated mesenteric bed (McGregor, 1965), the rat isolated

portal vein (Blair-West & McKenzie 1971) and in the cat isolated perfused terminal ileum (Turker, 1973). In some tissues, however, such as the rat isolated caudal artery (Nicholas, 1970), rat perfused hindlimb (Sato & Masuyama, 1971) and the dog's perfused paw (Liao & Zimmerman, 1972), angiotensin II potentiated responses to both endogenous and exogenous noradrenaline. Whereas the vascular tissues studied in Chapter I exhibited potentiation to endogenous noradrenaline only, exposure of the rabbit isolated aorta to angiotensin II induced a potentiation to both endogenous and exogenous noradrenaline.

Many theories have been proposed as to the mechanism of interaction of angiotensin II with the sympathetic nervous system. These have been discussed in Chapter I. They may be split broadly into two sections: those theories involving a pre-synaptic action of angiotensin II and those involving a post-synaptic action. It is reasonable to assume that in tissues in which angiotensin II potentiates responses to endogenous noradrenaline only, it is acting by a presynaptic mechanism such as facilitation of neuronal transmitter liberation, whereas in tissues in which responses to both exogenous and endogenous noradrenaline are potentiated either a presynaptic (e.g. inhibition of neuronal uptake of noradrenaline - Palaic & Khairallah, 1967a,b) or a postsynaptic (e.g. synergism at the α-receptor - Pals & Fulton, 1968)

site of interaction may be proposed.

On the rabbit isolated aorta, angiotensin II induced a non-specific form of potentiation rather than having a specific interaction with the sympathetic nervous system. This was not true of the other vascular tissues tested. This angiotensin II-induced sensitisation is via an action on its own specific receptors, as is the actual contraction induced by larger concentrations of the peptide on the aorta, as both actions are blocked by the analogue P-113 ($sarc^1$-ala^8-angiotensin II). This compound has been found to block, specifically, the contractor and potentiating actions of angiotensin II in vascular tissue (Rioux, Park & Regoli, 1973; Zimmerman, 1973). Therefore, the main body of this thesis comprises an attempt to discover the mechanism of this potentiating action of angiotensin II on responses to other vascular spasmogens in the rabbit isolated aorta, and possibly at the same time eliciting more information about the mechanism of the direct contractor effect of the polypeptide in the aorta.

The proposal that angiotensin II may act by inhibition of uptake of noradrenaline into sympathetic nerves (Palaic & Khairallah, 1967a, b) has received a great deal of attention in recent years, and is still controversial (see reviews by Lowe & Scroop, 1970; Gross, 1971; Starke, 1972). Therefore, it was

important in the present work to evaluate the possible contribution of this mechanism to the potentiation of responses to noradrenaline by angiotensin II on the aorta. The failure of maximally-effective neuronal noradrenaline-uptake blocking concentrations of cocaine, guanethidine and bethanidine to prevent a further increase in the response to noradrenaline on contact of the tissue with angiotensin II suggested, albeit indirectly, that angiotensin II was not inducing a potentiation of the responses to noradrenaline via an inhibition of neuronal uptake.

Tachyphylaxis to tyramine was reversed on incubation of the aorta with noradrenaline (4×10^{-6} g/ml). This was a much higher concentration than that used by Furchgott et al (1963) in their original experiments. This is probably due to the fact that aortae taken from reserpinised rabbits were used in the original study, and reserpine inhibits uptake of noradrenaline into the neuronal storage granules (Carlsson, Hillarp & Waldeck, 1963). As tyramine releases noradrenaline from the axoplasm itself rather than from storage granules (Harrison, Chidsey & Braunwald, 1963), a preparation with granular accumulation blocked would require less noradrenaline to reactivate the tyramine response. Angiotensin II did not prevent the noradrenaline-reversal of tyramine tachyphylaxis, whereas cocaine did. Studies involving radioactive noradrenaline confirmed that

angiotensin II did not inhibit uptake of noradrenaline into sympathetic nerve endings. As the potentiating action of angiotensin II was later found to be of a non-specific nature on the aorta, involving not just noradrenaline but a variety of vascular spasmogens, it is unlikely that angiotensin II would be acting by this mechanism.

Angiotensin II is inactivated by tissues in vitro and it is important, when negative results are obtained, with the peptide, to confirm that the biological activity is still intact. Palaic & Khairallah (1967 b) state that one way of avoiding a masking of the action of angiotensin II in uptake experiments is to give angiotensin II and noradrenaline simultaneously. This was done in the present experiments, with no significant effect on the uptake of noradrenaline in comparison with either control or angiotensin II-pretreated tissues. Also, Khairallah et al (1966a) noted that EDTA inhibited tissue angiotensinases and EDTA was present throughout the radioactivity experiments.

Khairallah et al (1966b) observed a potentiation of responses of the rabbit isolated aorta by indirectly-acting sympathomimetic amines. This did not occur with aortae taken from reserpinised animals, therefore, it appeared that release of endogenous stores of noradrenaline potentiated the response to other spasmogens. In the present experiments, the noradrenaline and 5-HT potentiating action of angiotensin II was the same in aortae taken from reserpinised rabbits as in controls.

The direct constrictor activity of angiotensin II also did not appear to involve an alteration of endogenous stores of noradrenaline, as it was unaffected by tyramine-blocking concentrations of cocaine and noradrenaline-blocking concentrations of phentolamine. Aortae taken from pre-reserpinised animals exhibited, in general, an increased responsiveness to the contractor action of angiotensin II, in agreement with the work of Sakurai & Hashimoto (1965). Also, tachyphylaxis to the direct constrictor action of angiotensin II on the aorta was not reversed on incubation with noradrenaline, an observation first reported by Khairallah et al (1966) on the rabbit isolated aorta.

Many compounds have been reported to increase the sensitivity of rabbit isolated aortae to vascular spasmogens via an increased cellular calcium content (for references, see Chapter IV), and the direct contractor action of angiotensin II on the aorta has been defined in terms of an increased intracellular free calcium ion level (Angles d'Auriac et al, 1972). However, the potentiation of responses to noradrenaline by angiotensin II was unaffected by exposure of the tissue to either calcium-free medium or to SKF-525A. Somlyo (1972) stated that supposedly "calcium-free" solutions are not necessarily calcium-free unless they contain a calcium-chelating agent, such as EDTA. However, in the present experiments, exposure to calcium-free (no EDTA) medium did prevent the poten-

tiation of responses to noradrenaline by 5-HT. Therefore, it appears that the potentiation of responses to noradrenaline by 5-HT is of a strongly calcium-dependent nature, possibly involving an increased influx of extracellular calcium ion into the cell.

Exposure of aortae to calcium-free + EDTA medium did block the angiotensin II-induced potentiation of responses to noradrenaline. This may be interpreted as indicating that angiotensin II does act by increasing influx of extracellular calcium, but this calcium is in a tightly bound form (e.g. to the extracellular muco-polysaccharide matrix) or that the membrane sodium-pump is involved. This latter possibility arises as Daniel (1965b) observed a blockade of the sodium/potassium pump in rabbit isolated aortae on incubation of the tissue in calcium-free + EDTA medium.

The effect of excess calcium in the medium bathing aortae - a blockade of the angiotensin II-induced potentiation of responses to noradrenaline, appeared to be a membrane-stabilising action, even though responses to noradrenaline were, themselves, unaffected. It did not support the idea that angiotensin II was increasing calcium influx, as it might be expected that an increased external concentration of calcium would increase calcium influx. However, the lack of effect of depolarising the membrane with potassium chloride suggests that angiotensin II was not acting simply by depolarising the membrane and hence making

the vascular smooth muscle cells of the aorta more responsive to drugs.

Contractor concentrations of angiotensin II induced a non-specific decrease in sensitivity of the aorta. This action was absent during exposure of the tissue to a calcium-free medium. The potentiation of aortic responses to noradrenaline by angiotensin II displayed a strong substrate (glucose)-dependency. Replacement of glucose by galactose did not significantly affect the percentage potentiation of noradrenaline by angiotensin II, whereas replacement with either fructose or sucrose almost abolished it. Altura & Altura (1970) found that exposure of rabbit isolated aorta to glucose-free Krebs solution for a 2 hr. period significantly reduced the contractor responses to angiotensin II, histamine and barium chloride, without significantly affecting those to either potassium chloride or catecholamines. They concluded that a failure of active transport mechanisms due to carbohydrate-substrate depletion might be involved.

The work of Wertheimer & Ben-Tor (1962), and Coe, Detar & Bohr (1968) suggests the existence of a selective sugar transport mechanism in, respectively, the aortae of rats and rabbits. Both groups of workers found results indicating a lack of transport of fructose. The results of the present experiments support this observation. Although other forms of metabolic

inhibition, O_2-lack and lowered temperature, had no significant effect on the percentage potentiation of noradrenaline by angiotensin II, the effectiveness of these procedures in the present experiments is in doubt (see Chapter V).

Thus, it appears that the potentiating action of angiotensin II is linked to an active transport process requiring readily available carbohydrate for energy. Angiotensin II induces a greater percentage potentiation at the lower end of the noradrenaline dose-response curve, suggesting that it is acting mainly at the level of the cell membrane (Somlyo & Somlyo, 1970).

Several membrane ion pumps have been described, including the sodium-potassium pump (Skou, 1965), the sodium-calcium pump (Bohr, Siedel & Sobieski, 1969) and the sodium "Pump II" (Hoffman & Kregenow, 1966). The last two pumps may be identical, as ethacrynic acid has been reported to inhibit both of them (Vincenzi, 1968).

The apparent lack of dependency on the presence of external calcium suggests that the sodium-calcium pump is not the site of action of angiotensin II in inducing its potentiation, although the direct contractor action of the polypeptide on the aorta is much more dependent on the presence of external calcium ions.

The inhibitory action of either exposing the aorta to ouabain or incubating in potassium-free solution, both well-known procedures to block the sodium-potassium pump (Garrahan et al, 1965), on the potentiation of noradrenaline by angiotensin II strongly suggests the involvement of this pump in the mechanism of action of angiotensin II. Ouabain potentiated the response to noradrenaline (and other agonists) whereas exposure to a potassium-free medium did not significantly affect it. Exposure of the tissue to ouabain increases the $(Na)^{+}_{I}$: $(Na)^{+}_{O}$ ratio, and hence the reactivity of the cell (Harris & Palmer, 1972) whereas potassium-free Krebs solution does not significantly alter this ratio, as although it blocks the efflux of sodium ions from the cell, it also blocks the influx; ouabain blocks the efflux only (Garrahan et al, 1965).

It has been noted by Daniel, Robinson, Kidwai, Wolowyk, Taylor & Paton (1971) that ouabain, on blocking the sodium-potassium pump of the rabbit aorta decreases the efflux of sodium ions, and as the sodium influx is not blocked the actual sodium content of the tissue is increased; hence the $(Na)^{+}_{I}$: $(Na)^{+}_{O}$ ratio is increased. It is possible that angiotensin II is also acting by this mechanism. However, increasing the extracellular sodium concentration, although increasing reactivity of the tissue to noradrenaline, did not, as may have been expected, increase the potentiating action of angiotensin II.

Harris & Palmer (1972) found that increasing the external sodium concentration by 5% actually increased the $(Na)_I^+$: $(Na)_O^+$ ratio of vascular smooth muscle cells, as the cell accumulated sodium ions. In the present experiments, this appeared to occur, as aortic reactivity was increased by an increased external sodium concentration. However, there was no further increase when low concentrations of angiotensin II (10^{-10} g/ml) were added to the bath. There must be a limiting value of sodium that the cell will accumulate and hence a limit to the $(Na)_I^+$: $(Na)_O^+$ ratio. Presumably as the sodium content of the cell is raised in a high sodium medium, then the resulting passive increase in influx of sodium ions into the cell on blockade of the sodium pump would be against a higher electrochemical gradient than with controls.

The mechanism of potentiation of responses to noradrenaline by ouabain and angiotensin II differs in the calcium-dependency. Exposure to a calcium-free medium blocked the potentiation of noradrenaline by ouabain, but not by angiotensin II. Therefore, on the basis of the sodium-calcium pump model the action of ouabain may be described as follows: ouabain inhibits the sodium-potassium pump, thus allowing an accumulation of intracellular sodium ions, and an increase in the $(Na)_I^+$: $(Na)_O^+$ ratio. This stimulates the sodium-calcium pump, and hence the efflux of sodium ions and the influx of calcium ions. The

increased intracellular free-calcium ion concentration in turn increases the reactivity of the vascular smooth muscle cell to other spasmogens (Somlyo & Somlyo, 1970).

To describe the action of angiotensin II, an assumption must be made, so that the description is, of necessity, hypothetical. Intracellular storage sites for calcium have been described in vascular smooth muscle (Bohr, 1973), and it is known that in contractor concentrations angiotensin II releases calcium bound to the cell membrane of the rabbit isolated aorta (Angles d'Auriac et al, 1972). If it is assumed that there is an intracellular sodium-calcium pump at the level of these storage sites, then an increased intracellular sodium concentration would increase the release of calcium from these sites and hence increase the reactivity of the cell via increased intracellular free-calcium levels. However, this hypothesis in itself does not explain the calcium-dependency of the action of ouabain, nor does it explain the observation that, in a calcium-free medium containing ouabain, the noradrenaline-potentiating action of angiotensin II is inhibited.

A possible explanation, in terms of cellular respiration, arises from the work of Swanson & Ullis, (1966). They observed a respiration-stimulating action of ouabain on isolated slices of rat cerebral

cortex. This was calcium-dependent, as in calcium-free medium ouabain induced a depression, rather than a stimulation of cellular respiration. Therefore, the potentiation of responses to vascular agonists by ouabain, and the calcium-dependency of this process, may involve a stimulation of cellular respiration either as well as or instead of an action via the sodium-calcium pump. It is feasible that this marked depression of cellular respiration by ouabain in calcium-free medium may also inhibit the potentiating action of angiotensin II on responses to noradrenaline possibly by reducing the activity of the hypothetical intracellular sodium-calcium pump.

The direct contractor action of angiotensin II on the rabbit isolated aorta was, like that to noradrenaline, potentiated in the presence of ouabain. However, while the contractor response to noradrenaline was not significantly affected on incubation of the tissue in a potassium-free medium, that to angiotensin II was reduced by an average of 50%. It appears, therefore, that blockade of the sodium-potassium pump itself does not inhibit the contractor response to angiotensin II, but inhibition of the passive influx of sodium ions does inhibit the response. Angiotensin II may actually potentiate itself by increasing the $(Na)^{+}_{I}$: $(Na)^{+}_{O}$ ratio of the responding cell. An increase of this ratio, during a contractor response of the rabbit isolated aorta to angiotensin II, has been observed

to increase the height of the response (Napodano et al, 1962). This phenomenon of autopotentiation to the response to angiotensin II on smooth muscle has previously been reported by Godfraind (1970), using successive doses of the polypeptide on isolated visceral smooth muscle.

In the present series of experiments, blockade of the sodium pump by either ouabain or potassium-free medium in isolated visceral smooth muscle (rat isolated colon) induces a non-specific decrease in sensitivity of the tissue, and a blockade of the spontaneous motility. On exposure to ouabain, the basal tone of the tissue was increased, possibly suggesting, as before, an increased intracellular sodium level, which in turn increased the intracellular free-calcium level. However, tissue reactivity was either decreased or unaffected, possibly as a result of the increased basal tone. Both procedures abolished the spontaneous contractions, suggesting an increased membrane stabilisation of the visceral smooth muscle cell.

As the responses to angiotensin II of both a direct and indirect nature appeared to be related to intracellular sodium concentrations, the effect of a diuretic on the responses of the pithed rat blood pressure to angiotensin II was examined. A previous report by Perez-Olea et al (1969) suggested that, in the pithed rabbit, frusemide had a specific anti-

angiotensin II action, although Blair-West et. al (1972) using the rat isolated portal vein, reported a non-specific blocking action of frusemide.

The pressor response to angiotensin II in the pithed rat appears to be direct, rather than via a release of noradrenaline. Schmitt & Schmitt (1968) reported that the pressor response to the polypeptide in the pithed rat was not mediated via the release of noradrenaline, nor altered by adrenalectomy. Finch & Leach (1969) showed that high doses of angiotensin II produced a biphasic pressor response in pentobarbital anaesthetised and pithed rats. Acute or chronic adrenalectomy did not alter the form of the response to the polypeptide in these preparations. These authors showed, however, that adrenergic neurone blockade with bethanidine or amine depletion with reserpine altered the rise to a simple rise. They concluded that large doses of angiotensin II were capable of indirectly stimulating sympathetic nerves.

In the present experiments, frusemide had a variable effect: pretreatment of the animals had no effect, and neither did intraperitoneal injection of frusemide. However, in just over half the animals tested, intravenous frusemide had a specific anti-angiotensin II action, while in the other half it had no effect. Increasing the dose in the "non-reactors" induced a non-specific depression.

Therefore, in the pithed rat frusemide does appear to have an inhibitory action on the constrictor response to angiotensin II in half of the population studied. It may be that there are "frusemide-sensitive" and "frusemide-insensitive" animals. It has previously been reported by Finch (1971) that frusemide treatment had no effect on the resting blood pressure of normotensive rats, but reduced that of hypertensive rats. In this experiment the animals responded uniformly to frusemide - there were no "sensitive" and "insensitive" animals.

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