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INFLUENCE OF ANGIOTENSIN, VASOPRESSIN OR CHANGES IN FLOW RATE ON VASOCONSTRICTION, CHANGES IN VOLUME AND [^{3}H]-NORADRENALINE RELEASE FOLLOWING POSTGANGLIONIC SYMPATHETIC NERVE STIMULATION IN THE ISOLATED CAT SPLEEN

BY

G. HERTTING AND J. SUKO

From the Institute of Pharmacology, University of Vienna, Vienna, Austria

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Feldberg & Lewis (1964) have shown that the polypeptides angiotensin and bradykinin release catecholamines from the adrenal medulla *in situ*. Especially angiotensin was in this respect a remarkably potent compound, since it released, on a molar basis, a thousandfold amount of catecholamines. Vogt (1965) confirmed this finding, using isolated perfused suprarenals ; in this preparation, however, angiotensin was considerably less effective.

Several authors have suggested that angiotensin releases also noradrenaline from its peripheral stores (Beaulnes, 1963 ; Youmans, Davis, Krasney, Paudler, Smith & Pua, 1964 ; Krasney, Paudler, Smith, Davis & Youmans, 1965 ; Liebau, Distler & Wolff, 1965) or increases the noradrenaline output of sympathetic stimuli (Benelli, Della Bella & Gandini, 1964).

Some observations supported this hypothesis: Dichloroisoproterenol, a β-receptor blocking compound, partially blocked the positive inotropic and chronotropic actions of angiotensin (Kuschinsky & Lüllmann, 1959 ; Beaulnes, 1963). Noradrenaline pretreatment prevented the tachyphylaxis of angiotensin (Beaulnes, 1963 ; Liebau *et al.*, 1965). The effects of stimulating the sympathetic nerve of the spleen were potentiated by angiotensin (Benelli *et al.*, 1964). It was also suggested that the stimulation of central sympathetic centres may contribute to the actions of angiotensin (Bickerton & Buckley, 1961).

Koch-Weser (1964a) on the other hand did not find any inhibition of the positive inotropic effect of angiotensin on the isolated papillary muscle by dichloroisoproterenol. Koch-Weser (1964b) and Fowler & Holmes (1964) found the myocardial actions of angiotensin unaltered in hearts which were depleted of their noradrenaline content by reserpine pretreatment.

In view of these conflicting findings it seemed desirable to investigate whether angiotensin itself has any direct noradrenaline-releasing properties or influences the noradrenaline release by sympathetic nerve stimulation.

METHODS

Spleen perfusion

Spleens of cats (both sexes, 2–3 kg) were isolated and placed in a plethysmograph. They were perfused with Krebs-Ringer bicarbonate solution (NaCl 6.92 g, $NaHCO_3$ 2.10 g, KCl 0.35 g, $MgSO_4 .7H_2O$ 0.29 g, KH_2PO_4 0.16 g, $CaCl_2$ 0.28 g, glucose 1.15 g/l.) saturated with a mixture of 95% and carbon dioxide 5%. Ascorbic acid (25 mg/l.) was added to the perfusion solution to prevent oxidation of noradrenaline. Preliminary experiments showed that this concentration of the ascorbic acid does not interfere with the performance of the organ and is sufficient to counteract the breakdown of the noradrenaline for at least 15 min in the oxygenated solution. The bath as well as the perfusion fluid was kept at 37° C. The perfusion experiments were performed either using a pump to obtain a constant flow rate or using a mariotte bottle to maintain a constant perfusion pressure.

The perfusion pressure was measured with a mercury manometer, the changes in volume were recorded with a Barcroft recorder on a kymograph. The plethysmograph and the recording system were calibrated after each experiment. The venous cannula was raised in order to obtain a venous resistance of 4 cm H_2O, which corresponds to the physiological pressure in the portal vein. It is important to have a resistance in the outflow to maintain a constant splenic volume.

The flow rate was measured by collecting the splenic outflow at minute intervals in graduated tubes.

Denervated spleen

Chronic postganglionic sympathetic denervation was performed by transection of the splenic nerve fibres during nembutal anaesthesia under aseptic conditions at least 10 days prior to the perfusion experiment. The fact that the noradrenaline content of the spleen (normally 2.5 μg/g) fell to less than 0.02 μg/g was proof of the effective denervation of the organ.

Reserpine pretreatment. 2 mg/kg reserpine (Serpasil CIBA) was given to cats intramuscularly 72 and 48 hr prior to the perfusion. The reserpinized animals were kept in a room at 32° C. The noradrenaline content of the spleen of these reserpinized cats fell to less than 0.1 μg/g.

[^3H]-noradrenaline pretreatment

In some experiments 125 μc/kg of dl-7[^{3}H]-noradrenaline (New England Nuclear Corporation, Boston, Mass., spec. activity 20 μc/μg) was given intravenously to cats 6 hr prior to perfusion of the spleen. In these experiments the splenic effluent was collected over 1 min periods in graduated tubes containing 0.2 ml. perchloric acid (30%), 0.2 ml. ascorbic acid (2%) and 0.1 ml. EDTA (5%) per 10 ml. perfusate.

Noradrenaline assay

The noradrenaline content of the spleen was determined by homogenizing the organ in 10 volumes of 0.4 N perchloric acid.

The endogenous as well as the [^{3}H]-noradrenaline was isolated by absorption on aluminium oxide (Woelm, Eschwege; neutral) at *p*H 8.5 and elution with 0.25 N HCl. Endogenous noradrenaline was assayed spectrofluorometrically by the method of Crout, Creveling & Udenfriend (1961). The eluates containing the [^{3}H]-noradrenaline were evaporated in vacuo, taken up once in 1 ml. methanol and twice in 2 ml. ethanol and the washings transferred to counting vials.

The radioactivity was assayed in a liquid scintillation spectrometer, quenching was corrected in all samples by adding an internal standard.

Splenic nerve stimulation

The splenic nerve fibres were carefully dissected and placed on bipolar platinum electrodes and stimulated using a Grass stimulator, model S 4 B, with 10 V for a duration of 2 msec stimulus and a frequency of 30/sec. 150 stimuli were given in each stimulation period.

Drugs used

Angiotensin (Hypertensin CIBA), vasopressin (Pitressin Parke, Davis & Co) or tyramine-HCl were injected in a volume of 0.3 ml. during 10 sec into the arterial cannula.

RESULTS

Effects of the injection of angiotensin, vasopressin or tyramine into an isolated perfused cat spleen

In these experiments a constant flow rate was used for perfusion of the organ. Angiotensin (0.3 μg) produces a remarkable contraction of the spleen as well as a vasoconstriction as seen by the increase of the perfusion pressure. There was no increase whatever in the [^{3}H]-noradrenaline output of the spleen after administration of angiotensin. However, the spontaneous release of [^{3}H]-noradrenaline diminished in the later periods.

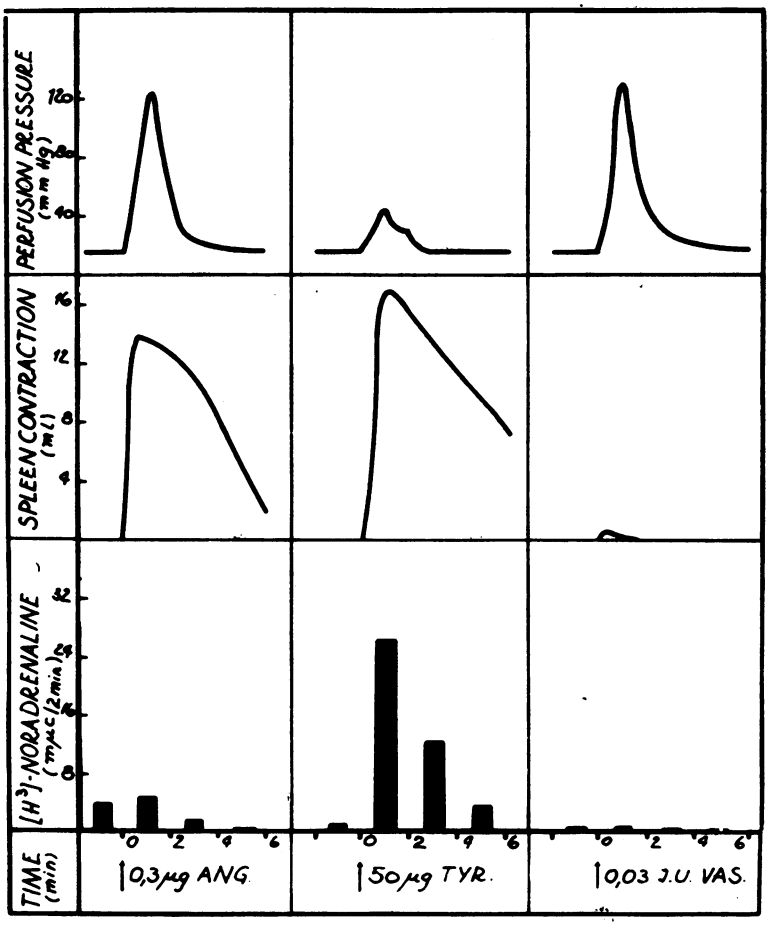

Fig. 1. Effects of injecting angiotensin, tyramine or vasopressin into an isolated perfused cat spleen. The animal received 125 μc/kg dl-7-[^{3}H]-noradrenaline intravenously 6 hr prior to the perfusion. The spleen was perfused at a constant flow rate of 6 ml./min with Krebs-Ringer bicarbonate solution. Perfusion pressure in mm Hg, spleen contraction in ml. volume change and liberated [^{3}H]-noradrenaline in mμc/2 min are shown. Angiotensin (ANG.) 0.3 μg, tyramine (TYR.) 50 μg and vasopressin (VAS.) 0.03 i.u. were injected intra-arterially at the arrows (↑). [^{3}H]-noradrenaline determinations were made in two minute samples of the perfusion fluid.

Vasopressin caused vasoconstriction only; the splenic volume and the [^{3}H]-noradrenaline output remained practically unchanged.

Tyramine, as an indirectly acting sympathomimetic amine, acts by releasing noradrenaline from its stores (Burn, 1958). As seen in Fig. 1, the contraction of the spleen after tyramine injection coincides with a release of [^{3}H]-noradrenaline. The vasoconstriction is less pronounced than that found with angiotensin.

Effects of the injection of angiotensin, vasopressin or tyramine into an isolated perfused cat spleen after chronic postganglionic sympathetic denervation or reserpine pretreatment

A constant flow rate was also used in these experiments for perfusion of the organ. The injection of tyramine had no effect in spleens deprived of noradrenaline either by reserpine pretreatment or by chronic postganglionic denervation (Fig. 2). In contrast to this the effects of angiotensin and vasopressin on the changes in volume and vasoconstriction were unaltered by these procedures.

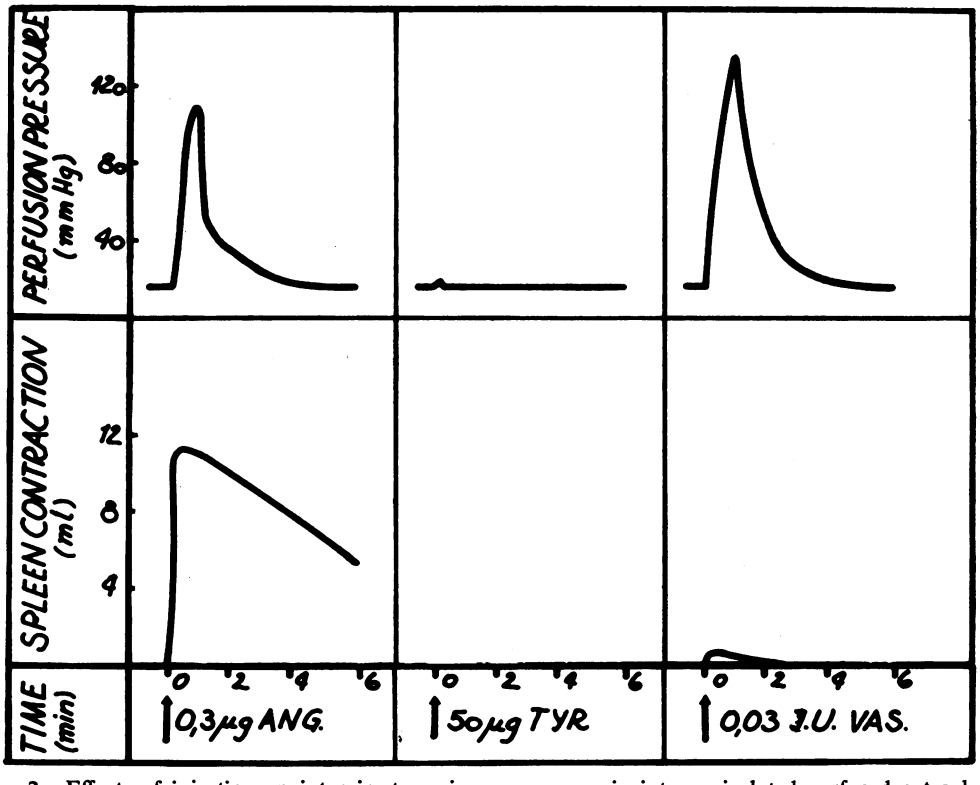

Fig. 2. Effects of injecting angiotensin, tyramine or vasopressin into an isolated perfused cat spleen 14 days after postganglionic sympathetic denervation. The spleen was perfused at a constant flow rate of 6 ml./min with Krebs-Ringer bicarbonate solution. Changes in perfusion pressure (mm Hg) and changes in spleen volume (ml.) are shown. Angiotensin (ANG.) 0.3 µg, tyramine (TYR.) 50 µg and vasopressin (VAS.) 0.03 i.u. were injected intra-arterially as indicated by the arrows (↑).

Influence of the injection of angiotensin or vasopressin upon the effects of nerve stimulation of the isolated cat spleen.

The organs were perfused by constant pressure. Stimulation of the sympathetic nerves caused noradrenaline release which resulted in contraction of the spleen. If the nerve is repeatedly stimulated, usually the amount of noradrenaline released in each stimulation period tends to decrease slowly. Angiotensin injected in doses of 0.025 μg to 0.05 μg produced a small contraction of the spleen, followed by a decreased flow rate. Stimulation of the nerve at this moment produced a much larger volumetric change than seen in control periods, but the amount of [^{3}H]-noradrenaline released by stimulation of the nerve remained unchanged or only slightly increased.

Vasopressin injected into the arterial cannula produced solely a decrease in flow rate. Neural stimulation at this point did not result in a considerably higher release of [^{3}H]-

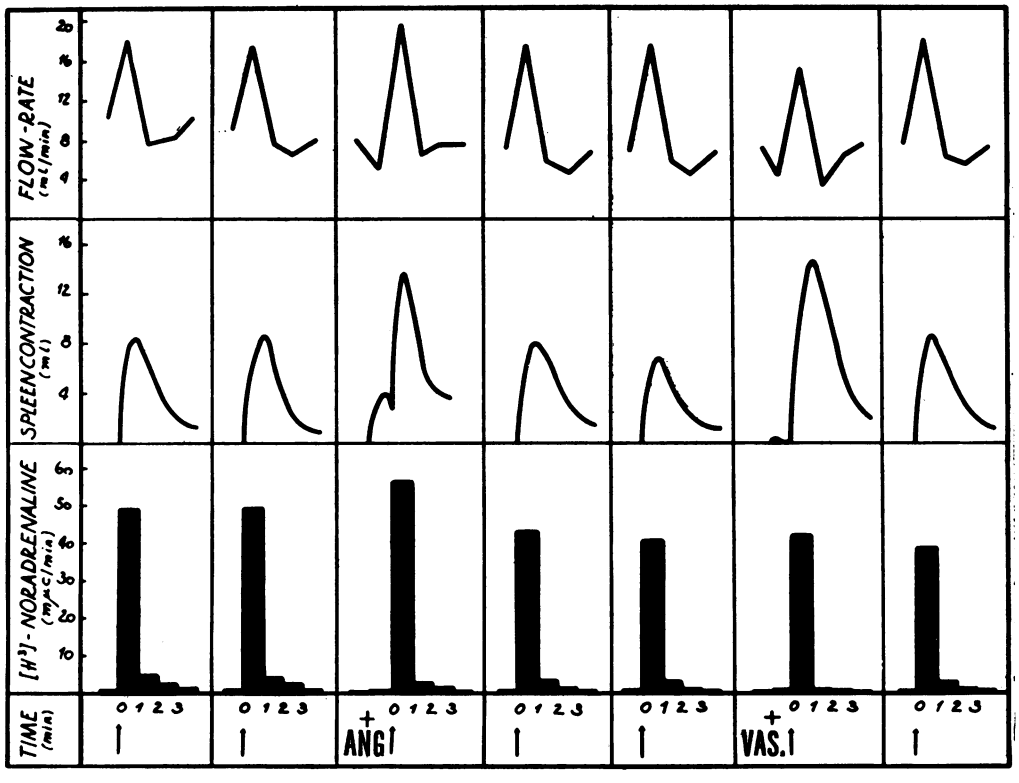

Fig. 3. Influence of the injection of angiotensin or vasopressin on the effects produced by nerve stimulation in an isolated perfused cat spleen. The animal received 125 μc/kg dl-7-[^{3}H]-noradrenaline i.v. 6 hr prior to the perfusion. The spleen was perfused at a constant pressure of 40 mm Hg with Krebs-Ringer bicarbonate solution. At each arrow (↑) the splenic nerve was stimulated with rectangular pulses of 2 msec duration at 10 V, and a frequency of 30/sec for 5 sec. Angiotensin (ANG.) 0.025 μg–0.05 μg was injected 1 min 45 sec and vasopressin (VAS.) 0.04 i.u. 1 min prior to the stimulation of the nerve as indicated (+). Flow rate (ml./min), spleen contraction in ml. volume change and liberated [^{3}H]-noradrenaline in mμc/min are shown.

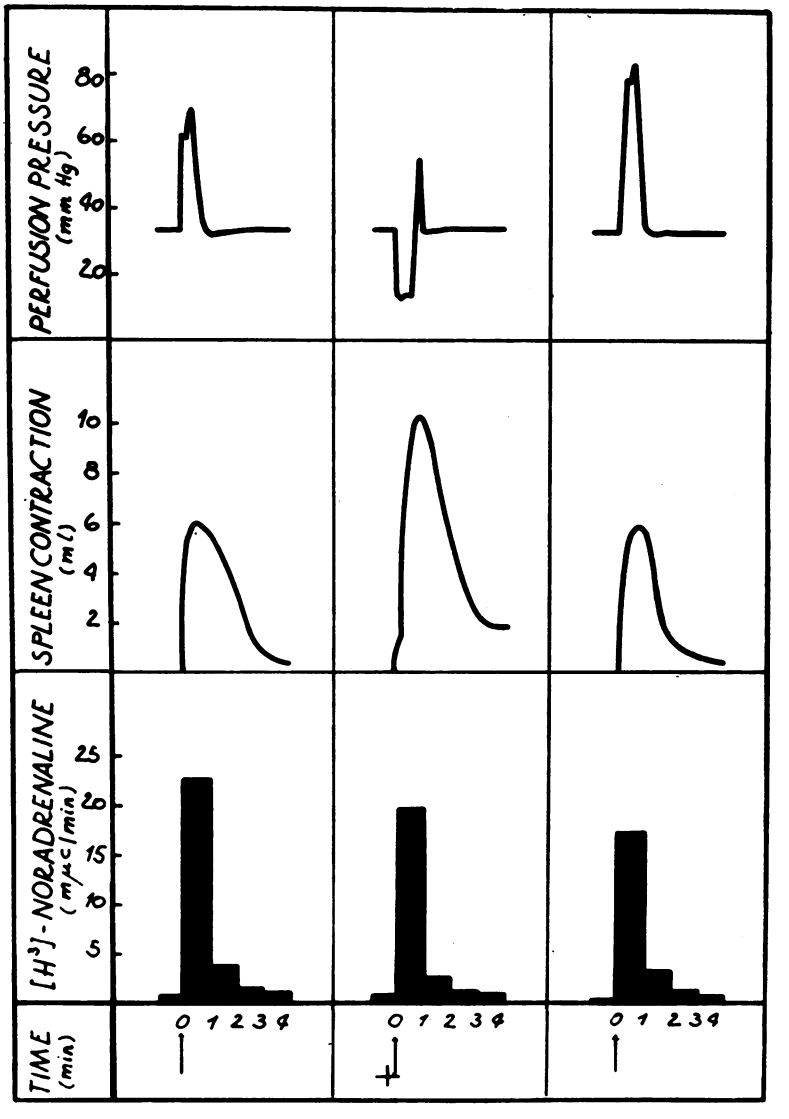

Fig. 4. The effect of stopping the perfusion prior to electrical stimulation of the splenic nerve on the splenic contraction and [^{3}H]-noradrenaline release. The animal received 125 μc/kg dl-7-[^{3}H]-noradrenaline intravenously 6 hr prior to the perfusion. The organ was perfused at a constant flow rate of 11.5 ml./min with Krebs-Ringer bicarbonate solution. At each arrow (↑) the splenic nerve was stimulated with rectangular pulses of 2 msec duration at 10 V, and a frequency of 30/sec for 5 sec. 15 sec before the second stimulation the flow was stopped (+) for 45 sec. Perfusion pressure (mm Hg), volumetric changes of the spleen (ml.) and [^{3}H]-noradrenaline output (mμc/min) are shown.

noradrenaline than that obtained with control stimulations; the splenic contraction, however, appeared markedly potentiated.

Several experiments were performed in the same manner and basically the same results were obtained: in all cases, the administration of angiotensin or vasopressin followed by stimulation of the splenic nerve caused a marked increase in splenic contraction, whereas the [^{3}H]-noradrenaline output was virtually unchanged. There was no relationship, between the increase in the contraction of the spleen and the increase of [^{3}H]-noradrenaline output.

In almost all cases the concentration of [^{3}H]-noradrenaline (=[^{3}H]-noradrenaline/ml. outflow) in the perfusate of the stimulation periods after the angiotensin or vasopressin was higher than in the equivalent stimulation periods, without the pre-treatment with the vasoconstrictor agents.

The effect of stopping the perfusion prior to electrical stimulation of the splenic nerve on the splenic contraction and [^{3}H]-noradrenaline release

To determine whether the rate of the inflow had any influence on the size of the splenic contraction the following experiment was performed. Spleens were perfused at a constant flow rate of 10–12 ml./min and the splenic nerve was electrically stimulated every 15 minutes as in the previous experiments, the volumetric change, perfusion pressure and the [^{3}H]-noradrenaline released were recorded.

Shortly before neural stimulation the flow diminished or completely stopped and in all cases the subsequent nerve stimulation produced a larger contraction of the spleen than found under the control conditions. The [^{3}H]-noradrenaline released was practically the same as in the control periods. Figure 4 shows such an experiment in which the flow was stopped 15 sec prior to the stimulation and restarted after 45 sec.

DISCUSSION

The actions of tyramine are mediated by the noradrenaline which it releases from its stores. In the organs where the sympathetic nerve endings had been destroyed by postganglionic sympathetic denervation, or which did not contain any noradrenaline on account of pretreatment with reserpine, tyramine ceased to produce any action at all, even if higher doses were employed.

Angiotensin injected into the artery of an isolated perfused cat spleen produces vasoconstriction as well as a contraction of the spleen. If these actions, at least in part, are mediated by the release of noradrenaline, this catecholamine should be found in the perfusate. Moreover, the actions of angiotensin should be diminished in those experiments where the spleens were deprived of noradrenaline by chronic postganglionic sympathetic denervation or reserpine pretreatment. In our experiments there was no trace of an increased noradrenaline output following an injection of angiotensin or vasopressin. Moreover, both polypeptides were fully active in spleens which had practically no noradrenaline.

Nash (1963) already has shown that the pressor action of vasopressin is not altered in reserpinized animals. In the spleen, vasopressin contracts only the smooth muscles of the blood vessels, whereas it is without effect on the capsular and the trabecular smooth muscles (De Boer & Carrol, 1924). In our experiments the vasoconstriction following an injection of vasopressin was of the same magnitude in the normal spleen as in chronically denervated or reserpine-pretreated organs. It can therefore be concluded that neither angiotensin nor vasopressin acts in an isolated spleen by producing a release of noradrenaline.

Noradrenaline released from its stores by nerve stimulation or administered by injection partly acts on the receptors, partly leaves the spleen via the circulation and partly is taken up and bound at the nerve endings ; a small fraction is metabolized. In a perfused normal spleen the amount of noradrenaline removed during a single passage is about 60% of the noradrenaline administered (Hertting, Suko & Widhalm, 1965b). Therefore changes in the rate of uptake produce very marked changes in the amount of noradrenaline leaving the organ. The concentration of noradrenaline acting on the receptor sites is the limiting factor for the duration and the extent of its action.

Compounds such as cocaine (Whitby, Hertting & Axelrod, 1960 ; Muscholl, 1961 ; Hertting, Axelrod & Whitby, 1961b ; Thoenen, Huerlimann & Haefely, 1964) or sympathetic denervation (Hertting, Axelrod, Kopin & Whitby, 1961a ; Hertting & Schiefthaler, 1964) interfere with the uptake, thus increasing the concentration of noradrenaline acting on the receptor sites and producing supersensitivity.

It was now the question whether the potentiation of the effects of nerve stimulation produced by angiotensin or vasopressin could be explained on this basis. However, the total amount of noradrenaline obtained in the perfusate during the stimulation period following the administration of these compounds is not greater, or only slightly greater, than that obtained during control periods even if the splenic contraction following the nerve stimulation appears to be considerably increased.

We have shown previously that changes in the flow rate influence the rate of uptake in a perfused spleen (Hertting & Schiefthaler, 1963 ; Hertting, 1965a). In an organ perfused at a low flow rate the noradrenaline stays longer in the vicinity of the binding sites and a greater amount is taken up and bound. Stimulation of the splenic nerve is usually followed by contraction of the spleen. Hence some of the released noradrenaline is discharged rapidly despite a decreased flow rate produced either by the introduction of vasoconstrictor compounds or decreased perfusion pressure. If, however, the splenic contraction produced by stimulation of the nerve is prevented by an adrenergic α-receptor blocker and at the same time the flow reduced to a minimum by the injection of angiotensin or vasopressin, practically no noradrenaline leaves the spleen following this stimulation.

It can not be completely ruled out that angiotensin does not interfere with the uptake mechanism at all, but the experiments performed with the presence of an α-receptor blocker show that this action can be only of minor importance.

The size of a spleen perfused under our experimental conditions depends on several factors. If the inflow and the outflow are in equilibrium at a given constant peripheral resistance and perfusion pressure, the perfused organ has reached a certain volume.

During and after nerve stimulation the spleen contracts and discharges an extra amount of fluid in addition to the normal outflow; the quantity depending on the duration and intensity of stimulation. At the same time, since there is only a small and short-lasting increase in vascular resistance due to nerve stimulation, a certain amount of fluid enters the spleen via the artery. Since we are registering changes in volume our volumetric changes are, in fact, the outflow minus the inflow. Since the spleen contraction is a three-dimensional function the simple recording of changes in length (Benelli *et al.*, 1964) is also influenced by the same factors.

If the inflow is stopped or diminished either mechanically or by means of vasoconstrictor agents shortly before nerve stimulation a bigger volumetric change is recorded during nerve stimulation although the actual amount of fluid discharged from the spleen remains unaltered or decreases.

The same mechanism may serve as an explanation for the increased amount of noradrenaline observed to leave the spleen in periods when vasoconstrictor compounds were given prior to the nerve stimulation.

The amount of fluid discharged in periods of decreased inflow is equal to or even smaller than the amount discharged in control periods, but represents a larger aliquot of the distribution space of released noradrenaline. Therefore a greater part of noradrenaline which was released upon nerve stimulation is discharged from the spleen. Of course the amount of noradrenaline discharged is influenced by the relationship between the distribution space, the amount of fluid discharged and the inflow. Hence, all variations between increased, unchanged or even decreased noradrenaline output can be found.

Concomitantly, the concentration of noradrenaline in samples of smaller volumes is increased. It can be assumed that the increased noradrenaline concentration in the perfusate reflects the situation at the receptors. This may influence, in addition to the mechanisms discussed above, the development of the increased response of the spleen to nerve stimulation which occurs after the decrease in inflow.

The size of the spleen contraction and the amount of noradrenaline leaving the spleen following a nerve stimulation depend to a large extent on the rate of the inflow during and after the nerve stimulation. It is therefore essential to know whether the perfusion is performed under constant perfusion pressure or constant perfusion flow rate. Vasoconstriction diminishes the inflow into the spleen to a much greater extent and for a longer period of time in an organ perfused by a constant perfusion pressure. If perfused by constant perfusion rate, the perfusate is forced by the pump into the organ so that the quickly rising pressure overcomes the vasoconstriction. Our experiments were performed using a purely postganglionic sympathetic nerve preparation. The results, therefore, do not exclude the possibility that angiotensin has a stimulatory effect on sympathetic centres of the central nervous system (Nishith, Davis & Youmans, 1962; Benetato, Haulică, Uluitu, Babuianu, Mocodean, Stefănescu & Suhaciu, 1963) or facilitates the transmission of impulses in ganglia. Some of the effects of angiotensin in the whole organism may be produced by the catecholamines released from the adrenal medulla (Feldberg *et al.*, 1964). Shortly before finishing our manuscript a paper of Thoenen, Hürlimann & Haefely (1965) was published. These authors also performed experiments using the isolated perfused spleen preparation. They examined the effects

of angiotensin on the removal of infused noradrenaline by the spleen and the influence of angiotensin on the amount of noradrenaline released by nerve stimulation. An infusion of 10^{-8} g/min of angiotensin neither influenced the rate of removal of infused noradrenaline nor the amount of noradrenaline released by nerve stimulation although the increase in vascular resistance and the change in volume resulting from nerve stimulation were potentiated by angiotensin. The authors interpret this potentiation of the effects of nerve stimulation on splenic contraction and vascular resistance by a possible synergistic action of angiotensin and noradrenaline on the trabecular and vascular smooth muscles.

We were able to demonstrate that a potentiation of the change in volume of the spleen can be produced also by vasopressin, which has no action at all on the trabecular smooth muscles, as well as by a mechanically induced reduction in the flow rate. A mechanically induced reduction in flow produced effects on the splenic contraction similar to those seen with angiotensin or vasopressin. It may therefore be concluded that it is the decrease in flow rate which is primarily responsible for the potentiation by angiotensin of the change in volume of the spleen following nerve stimulation.

SUMMARY

1. The ability of angiotensin and vasopressin to release noradrenaline from its peripheral stores or to facilitate the noradrenaline release following sympathetic nerve stimulation has been investigated using an isolated, perfused cat-spleen preparation.

2. Angiotensin injected into the arterial cannula of the perfused spleen produced vasoconstriction and contraction of the spleen. Vasopressin showed vasoconstrictor properties only.

3. Neither angiotensin nor vasopressin released [^{3}H]-noradrenaline from the spleen. Tyramine was, as expected, a potent [^{3}H]-noradrenaline-releasing agent.

4. Both compounds were fully active in spleens following chronic, postganglionic sympathetic denervation or in reserpine-pretreated spleens which were deprived of their noradrenaline content. In these preparations tyramine was ineffective.

5. If angiotensin or vasopressin were injected into the spleen prior to electrical stimulation of the nerve, the resulting contraction of the spleen appeared to be increased. However, the amount of [^{3}H]-noradrenaline leaving the spleen was not considerably different from that obtained during control periods.

6. Similar effects, i.e., an increased change in volume with little or no change in noradrenaline release following nerve stimulation, were produced by reducing or stopping the inflow to the spleen shortly before applying the nerve stimulus.

7. It is suggested that angiotensin and vasopressin do not potentiate the effect of nerve stimulation by facilitating noradrenaline release or by preventing the rebinding of released transmitter, but that it is the decrease in flow rate which is responsible for this effect.

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