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ON VASODEPRESSOR ACTIVITY IN THE HYPOTHALAMUS*

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Abstract—Acetic acid extracts of hypothalamic tissue of pigs, cattle, and sheep were subjected to gel filtration on Sephadex and other purification procedures. Several vasodepressor fractions were obtained. The activity of one of these fractions was clearly due to oxytocin. However, another fraction, which is present in significant amounts in hypothalamic extracts of domestic animals, lowered the blood pressure of chickens and rats and relaxed isolated rat duodenum, but had no effect on isolated rat uterus. The vasodepressor property of this material was not abolished by treatment with thioglycolate, trypsin, pepsin, papain, chymotrypsin, carboxypeptidase, or leucine aminopeptidase. Data presented show that this fraction is not oxytocin, vasopressin, substance P, angiotensin, bradykinin, acetylcholine, 5-hydroxytryptamine, histamine, γ-aminobutyric acid, epinephrine, or norepinephrine. Many of its pharmacologic and chemical characteristics suggest that it is adenosine monophosphate. We suggest that in studies of hypothalamic extracts, it is unwise to equate vasodepressor activity with the presence of oxytocin.

DURING fractionation of hypothalamic tissue of ovine, bovine, and porcine origin in the search for hypophysiotropic hypothalamic releasing factors,[1, 2] two overlapping but clearly separated fractions were found to cause a fall in blood pressure in the chicken. Since it was known that only one of these fractions could be oxytocin, it was decided to investigate the characteristics of the other fraction. Although it has been known for many years that the hypothalamus is involved in the regulation of blood pressure,[3, 4] our interest was further increased by the recent report of Gorten *et al.*[5] that vasodepressor responses could be elicited in dogs by stimulation of the diencephalon.

EXPERIMENTAL

Materials and methods

Extraction. Fragments of beef, sheep, and pig hypothalamic tissue containing mainly the pituitary stalk–median eminence region and ventral hypothalamus, obtained within 15–30 min after the animals had been slaughtered, were frozen on dry ice and preserved at −20°. Treatment of this frozen or lyophilized hypothalamic tissue with acetone and petroleum ether was performed as described by Schally and Bowers.[6] This extraction probably removed most of the 5-hydroxytryptamine, epinephrine,

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norepinephrine, histamine, and acetylcholine,[7] in addition to the lipid materials. The tissue, defatted by treatment with acetone and petroleum ether, was extracted with 2 N acetic acid. The water-soluble extracts were heated to boiling, lyophilized, and re-extracted with glacial acetic acid.[6] A further purification was effected by gel filtration on Sephadex,[6, 8] with 0·1 M pyridine acetate or 1 M acetic acid as eluant. Peptide concentrations were measured by the Folin-Lowry method.[9]

Thin-layer chromatography was carried out in Desaga-Brinkman apparatus on plates prepared with cellulose and cellulose treated with fluorescent indicators.

Chemical treatment. Acid hydrolyses of samples of materials having vasodepressor activity were performed with 6 N hydrochloric acid at 105° for 20 hr. The hydrochloric acid was removed by repeated evaporation *in vacuo*. Thioglycolate reduction was performed by the method of Ames *et al.*,[10] except that the concentration of thioglycolate was increased from 0·01 to 0·05 M.

Enzymatic treatment. For enzymatic digestion with trypsin, chymotrypsin, carboxypeptidase, and leucine aminopeptidase, the samples were dissolved in 0·1 M ammonium acetate buffer, adjusted to pH 8·5, and incubated for 20 hr at 38°. The enzyme: substrate ratio was 1:15 to 1:20. Leucine aminopeptidase was activated by 0·2 mM $MgCl_2$. For hydrolysis with papain, the material was buffered to pH 5; 0·01 M sodium versenate was added, and the enzyme was activated by hydrogen sulfide. Incubation with pepsin was performed at pH 2 in 0·01 M hydrochloric acid.

Bioassays. Vasodepressor effects in the chicken were used as an assay according to Thompson's modification[11] of the procedure of Coon.[12] Blood pressure responses in the rat were also used for measuring vasodepressor effects. Materials were injected into the femoral vein, and arterial blood pressure was measured in the carotid as in the Dekanski assay.[13] In some instances, pretreatment of the rats with dibenzyline was omitted, to raise the arterial blood pressure. This resulted in greater sensitivity to vasodepressor peptides. Melanocyte-stimulating hormone (MSH) activity was measured by the photometric method of Shizume *et al.*, *in vitro*.[14]

For determination of oxytocic acitivity, the uterine horn of a virgin rat pretreated with diethylstilbestrol was suspended in de Jalon's solution.[15] Additional assays were performed with the proximal end of rat duodenum suspended in de Jalon's solution.[16]

For the vasopressor and vasodepressor assays, recordings were made with a Physiograph (E and M Instrument Co., Houston, Texas) equipped with a Statham physiological transducer. An isotonic myograph transducer was employed for the smooth-muscle assays. Samples were assayed at a neutral pH before and after lyophilization.

RESULTS

Since the vasodepressor assay of unfractionated extracts would measure both oxytocin and other vasodepressor substances, hypothalamic extracts of porcine origin were purified on Sephadex G-25. Figure 1 shows the chromatographic pattern and biological activities observed during molecular sieving through gel filtration on Sephadex. There was good separation of the extracts into several peaks, distinct chemically and biologically. Three areas with MSH activity, indicated in order of decreasing molecular weight, are ACTH, β-MSH and α-MSH.

The peak of lysine vasopressin and oxytocin (tubes 72–80) emerged together, owing to similarity in their molecular weights (1054 and 1006). Samples of this fraction

lowered blood pressure in chickens and contracted rat uterus. Aliquots of fractions 84–88 and 89–96 produced a similar fall in blood pressure in the chicken (Fig. 2) and in the rat (Fig. 3). The responses increased as the doses were increased. However-even at dosages 6 times that giving a vasodepressor response (19 times that for sheep extract), these fractions had no effect on the isolated rat uterus. The uterine and vaso-

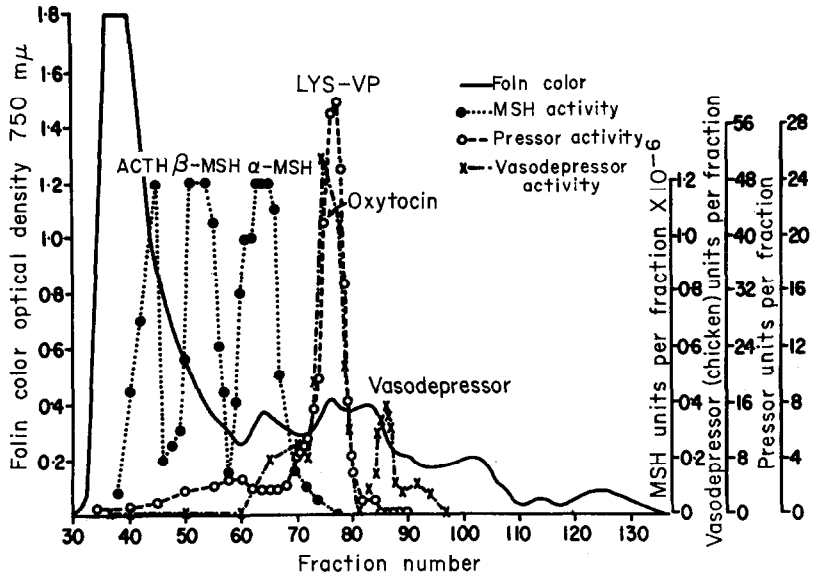

FIG. 1. Gel filtration of pig hypothalmic extracts on a column of Sephadex G-25 (4·3 × 160 cm) in 1 M acetic acid. Fraction size 25 ml; 50-μl aliquots taken for Folin Lowry analyses.

depressor activity of the oxytocin fraction (tubes 72–80) was abolished by incubation with thioglycolate. The vasodepressor effect in the chicken and the rat found in fractions 84–89 and 89–96 was not affected by thioglycolate.

Similar results were obtained when fractionation was performed in 0·1 M pyridine acetate pH 5 instead of 1 M acetic acid, or when a column 8 × 200 cm was used instead of the column described. Fractionation of beef and sheep hypothalamic extracts led to the same results. In all cases a material emerged after oxytocin which had vasodepressor activity in the chicken and the rat, but which had no uterine-contracting activity. In some cases the vasodepressor activity in the rat was obscured by traces of vasopressin, which trail into that area.

When the preparations of vasodepressor material were hydrolyzed with 6 N HCl, the vasodepressor effect on blood pressure in chickens and rats was abolished. Similarly treated preparations of histamine were still fully active after hydrolysis. Incubation for 20 hr with trypsin, chymotrypsin, leucine aminopeptidase, carboxy-peptidase, papain, or pepsin did not abolish the blood pressure effect in rats or chickens.

γ-Aminobutyric acid was easily differentiated from the vasopressor material, since at a dosage of 5 μg it caused an elevation in blood pressure in the chicken. Atropine did not abolish the vasodepressor effect in the rat.

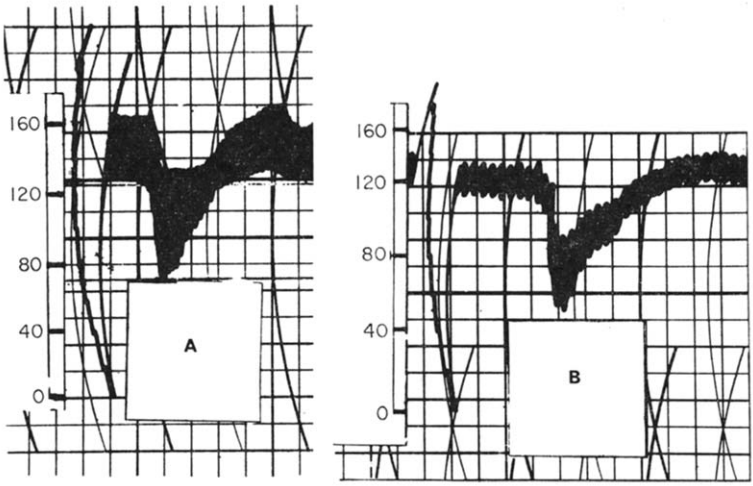

FIG. 2. Effect of vasodepressor material of porcine origin on arterial blood pressure of pentobarbital-anesthetized chicken. Ordinate: blood pressure in mm Hg. Abscissa: time in min (chart speed 0·05 cm/sec). Dosage: 2000 μg material purified from an equivalent of 2·5 pig hypothalamic fragments. A, Hypothalamic vasodepressor; B, adenosine-5′-phosphate (50 μg).

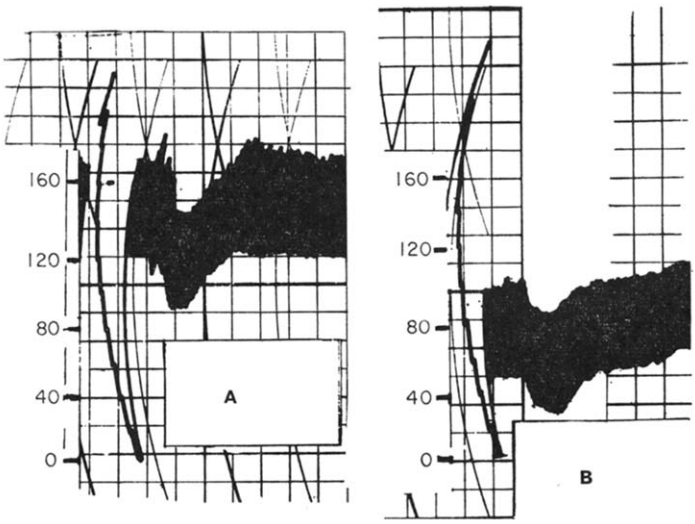

FIG. 3. Effect of vasodepressor material of porcine origin on arterial boood pressure of urethane-treated rat. Ordinate: blood pressure in mm Hg. Abscissa: time in min (chart speed 0·05 cm/sec). Dosage: 800 μg material purified from an equivalent of one pig hypothalamic fragment. A, Hypothalamic vasodepressor; B, adenosine-5′-phosphate (20 μg).

The hypothalamus is known to contain a high concentration of Euler's substance P.[17] Although the chemical characteristics of substance P and our vasodepressor material seemed different (18–20), their effects were measured on rat duodenum, which contracts on exposure to substance P.[16] The vasodepressor material caused only relaxation of the rat duodenum, in contrast to histamine, acetylcholine, and 5-hydroxytryptamine.

Other substances found in hypothalamic extracts are nucleotides. Adenosine-5′-phosphate and adenosine-3′-phosphate lowered blood pressure in chickens and rats at doses as low as 50 μg and 20 μg respectively. The patterns of onset and duration of responses were similar to those produced by the hypothalamic vasodepressor (Figs. 2 and 3). Adenine was inactive as a vasodepressor at doses as high as 2 mg. Other nucleotides such as guanosine-5′-phosphate and cytidine-5′-phosphate were also inactive. The behavior of the hypothalamic vasodepressor material on Sephadex, suggesting a small molecular weight, and its strong anodic mobility on free-flow electrophoresis, were identical with that of adenosine-3′- or -5′-phosphates. In addition, the vasodepressor material exhibited strong u.v. absorption spectra characteristic of nucleotides and showed titration curves similar to those given by nucleotides. The confirmation that our compound was a nucleotide was obtained by a positive molybdenum-blue test after chromatography. Furthermore, thin-layer chromatography, in systems of n-butanol:acetic acid:water (4:1:5) and 1 M ammonium acetate (pH 7·5):95% ethanol (3:7) showed spots with identical R_f as adenosine-5′-phosphate. Figure 4 shows the behavior of hypothalamic vasodepressor and adenosine-5′-phosphate on thin-layer chromatography.

We noted, however, that after purification on Sephadex and free-flow electrophoresis, the active principle was still not homogeneous. There were also other materials present such as peptides (identified as free amino acids after acid hydrolysis), salts, and other nucleotides.

After the separation on Sephadex, bovine hypothalamic fractions, and to a lesser extent ovine and porcine materials, exhibited vasodepressor activity in the range corresponding to peptides with molecular weight of 2000–3000. These vasodepressor materials, when chromatographed on carboxymethylcellulose, displayed the characteristics of basic peptides.

DISCUSSION

Many substances are endowed with hypotensive properties. Some of them, like histamine, acetylcholine, and 5-hydroxytryptamine, are present in the hypothalamus.[7] These three compounds would most likely be eliminated by the extraction procedure.[7] Furthermore, histamine is not affected by acid hydrolysis, and atropine does not abolish the vasodepressor effect of our material.

Our material is clearly different from oxytocin in that it does not contract rat uterus and is not affected by thioglycolate. This latter finding indicates the absence of disulfide linkages. Abolition of activity by acid hydrolysis indicates the presence of acid-labile linkages. The results also indicate that it is incorrect to locate oxytocin in hypothalamic extracts on the basis of a vasodepressor response in the chicken alone.[18] Certain batches of hypothalamic extracts appeared to have this vasodepressor material as measured by the Coon assay[12] in quantity similar to that of oxytocin.

Substance P also exists in the hypothalamus, but its known chemical properties[19–21]

seem to differ from the vasodepressor material reported here. This material is also different from substance P since it relaxes rat duodenum, whereas substance P stimulates contraction. The biological activity of substance P is abolished by digestion with trypsin and chymotrypsin,[17] but this does not occur with our vasodepressor material.

This material also differs from vasopressin and angiotensin in that it lowers the blood pressure of rats instead of raising it as the other two peptides do. Bradykinin, which has not been reported to be present in hypothalamic extracts, is not affected by trypsin but is destroyed by chymotrypsin. Unlike the vasodepressor material, moreover, it stimulates contraction of isolated rat uterus.

Thus, the vasodepressor material reported here seems to differ chemically and pharmacologically from bradykinin, angiotensin, vasopressin, oxytocin, substance P, acetylcholine, histamine, 5-hydroxytryptamine, epinephrine, norepinephrine, and γ-aminobutyric acid. It clearly does not show the characteristic actions of these compounds: substance P, histamine, and acetylcholine stimulate rat duodenum; angiotensin, vasopressin, epinephrine, norepinephrine, and γ-aminobutyric acid raise blood pressure in the rat; oxytocin, bradykinin, angiotensin, 5-hydroxytryptamine, and acetylcholine stimulate rat uterus.

From its rate of migration through Sephadex, this vasodepressor substance appears to have a molecular weight smaller than 1000, although molecular size rather than weight governs the separation on Sephadex.[6] Moreover, some peptides that contain aromatic amino acids are retarded by adsorption and give an anomalously low molecular weight.[22]

It is of interest that this vasodepressor material is present in ovine and bovine hypothalamic extracts as well as in the porcine extracts. Galoyan recently purified from rat hypothalamus biologically active compounds, other than vasopressin and oxytocin, which affect coronary circulation and blood pressure.[23]

The physicochemical characteristics of our vasodepressor material (such as u.v. absorption, titration curves, behavior on Sephadex, thin-layer chromatography, and anodic mobility on free-flow electrophoresis) are similar to those of adenine monophosphates and related nucleotides. Like free adenine, which is formed by acid hydrolysis of a nucleotide, the acid hydrolysate of our material had no vasodepressor activity. Again like a nucleotide, the activity of the material was not affected by proteolytic enzymes or thioglycolate. Another resemblance is that both adenosine phosphates and our extract show vasodepressor activity in the chicken but do not stimulate isolated rat uterus.

If the vasodepressor material is indeed adenosine monophosphate, it is very unlikely that it plays a normal role in the control of blood pressure. It is of interest, nevertheless, that acetic acid extracts of hypothalamic tissue prepared under conditions precluding the hydrolysis of nucleic acids contain considerable amounts of this material. Its function remains to be established, but it may be noted that cyclic 3′,5′-adenosine monophosphate (3′,5′-AMP) has been implicated in many processes of endocrine or biochemical importance. Thus 3′,5′-AMP is involved in promoting glycogenolysis in tissue[24] and causes changes in permeability of the amphibian bladder similar to those caused by vasopressin.[25] Addition of 3′,5′-AMP to adrenal tissue is reported to stimulate the production of corticoids in the same manner as ACTH.[26] Cyclic 3′,5′-AMP also mimics the stimulatory effect of luteinizing hormone on steroido-

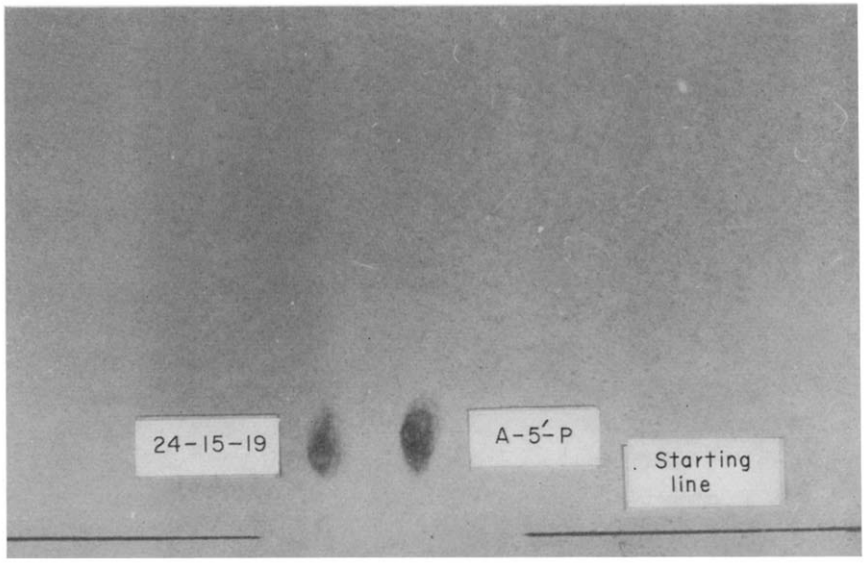

FIG. 4. Thin-layer chromatography of repurified hypothalamic vasodepressor 24-15-19 and adenosine-5′-monophosphate showing identical R_f's in a sytem of 1 M ammonium acetate (pH 7·5):95% ethanol (3 : 7). Color development with a specific reagent for adenine-containing compounds: potassium permanganate followed by chlorine gas resulting in yellow-orange spots which turned red after subsequent spraying with 3 N potassium hydroxide.

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genesis in bovine corpus luteum slices.[27] It is possible that if adenosine phosphate is present in the hypothalamus it may be involved in some neurosecretory processes necessary for the elaboration of hypothalamic releasing factors.[1, 2]

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