Biochimica et Biophysica Acta, 628 (1980) 201—208
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BBA 29161

ISOLATION, PURIFICATION AND AMINO ACID SEQUENCE OF A TRIPEPTIDE FROM BOVINE PINEAL TISSUE DISPLAYING ANTIGONADOTROPIC PROPERTIES

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(Received June 20th, 1979)

Key words: Tripeptide sequence; Antigonadotropin; (Bovine pineal tissue)

Summary

Studies were performed to isolate peptides displaying antigonadotropic properties from bovine pineal tissue. Inhibition of compensatory ovarian hypertrophy in adult mice was used as an index of activity to guide the purification of a bovine pineal extract in order to isolate these antigonadal peptides. Defatted bovine pineal glands were extracted with acetic acid and further purified by cation-exchange chromatography, gel filtration and paper electrophoresis. The electrophoretogram revealed sixteen ninhydrin spots, of which four were antigonadotropic. One of these fractions was subjected to paper chromatography which yielded two antigonadal fractions. Amino acid analysis of each of these fractions indicated that one was in pure form and the sequence was found to be threonylserinyllysine. The other fraction was heterogeneous, but contained no lysine. Analysis of the amino acid content of the other antigonadal fractions obtained after electrophoresis indicated the possibility that other peptides were present, but did not suggest the presence of arginine vasotocin.

Introduction

The mammalian pineal gland has been demonstrated to posses characteristics of an endocrine gland capable of suppressing reproductive function. Although

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Abbreviations: LH, luteinizing hormone; GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone.

the question as to what substance from the pineal is responsible for these antireproductive characteristics has not been fully resolved, two classes of compounds have emerged as candidates. One is the indoleamines, principally melatonin, which has been shown to mimic to some extent, the effects observed after pineal stimulation [1,2]. The other class of compounds is the peptides. Milcu et al. [3] isolated a pineal peptide which demonstrated characteristics similar to that of arginine vasotocin. The presence of this compound in the pineal was later confirmed by chemical means [4]. Subsequent studies with synthetic arginine vasotocin have characterized the compound to be an inhibitor of reproductive function in laboratory rodents [5—8].

When viewing the responsiveness of the reproductive endocrine system after pineal stimulation (i.e., by blinding or exposing the animal to a short photoperiod) and comparing these results to those brought about by either the indoles or arginine vasotocin, it is evident that something other than these compounds may be responsible for the biological activity. Indeed, other structurally unidentified compounds with antireproductive characteristics have been reported to be present in extracts of pineal tissue and are thought to be peptidic [9,10]. We have recently demonstrated the presence of more than one component in bovine pineal extracts devoid of melatonin and arginine vasotocin which displayed antigonadotropic characteristics [11,12]. In the present study we have attempted to further purify and isolate peptides from bovine pineal tissue which exhibit antireproductive properties.

Methods

Pineal crude extract. Fresh frozen bovine pineal glands (2 kg wet weight) were lyophilized and washed with 5 vols. acetone for 30 min at room temperature. The residue was filtered over vacuum, stirred with 1 vol. acetone for 15 min and refiltered. The defatted glands were then dried in a vacuum oven at 50°C for 3 h.

The glands were homogenized in 1.5 vol. 2 N acetic acid and stirred for 1 h at room temperature. The homogenate was centrifuged at 15 300 × *g* for 1 h and the resultant supernatant lyophilized. This fraction was mixed with 1 vol. glacial acetic acid, stirred for 45 min at room temperature and then centrifuged at 12 000 × *g* for 30 min. The supernatant was collected and diluted with 1 vol. distilled water and lyophilized. The material was redissolved in a small volume of 0.1 N acetic acid and centrifuged at 48 200 × *g* for 1 h. The supernatant was collected and lyophilized yielding 7.09 g of material which was then further purified by ion-exchange chromatography.

Ion-exchange chromatography. A column (1.5 × 30 cm) of analytical grade polystyrene cation-exchange resin (Dowex [H^+]) was used. The lyophilized material of the acetic acid extract was dissolved in 0.06 M acetic acid (pH 3.0) and placed on the bed of resin. Elution was carried out with successive washes of H_2O, 0.2 M pyridine (pH 4.0), 0.2 M pyridine (pH 5.0) and 1 M pyridine (pH 8.5) at room temperature. Each of the eluates was collected and lyophilized.

Gel filtration. The lyophilized fraction obtained after ion-exchange chromatography was dissolved in 1% aqueous NH_4HCO_3 and placed on a (1.5 × 80 cm) column of Sephadex G-25 (fine) equilibrated with 1% NH_4HCO_3. The material

was eluted with 1% NH_4HCO_3 and monitored at 206 and 254 nm continuously by a flow-through ultraviolet monitor (flow rate, 45 ml/h, 4.0-ml fractions). Fractions were pooled based on the elution profile and lyophilized. Protein content of each fraction was determined by the method of Lowry et al. [13].

Paper electrophoresis. The lyophilized fraction obtained by gel filtration was further purified by paper electrophoresis. Paper electrophoresis was carried out in Versol-cooled tanks (Sarvant Instrument) at 65 V/cm at 15°C. The lyophilized fraction was dissolved in H_2O and spotted on the paper and run for 30 min in pyridine/acetic acid/H_2O (100 : 4 : 900, by vol., pH 6.5). Guide strips containing a standard amino acid mixture were stained with ninhydrin containing cadmium acetate [14]. Materials on the paper were eluted with water and subsequently lyophilized.

Paper chromatography. The electrophoretic fraction was dissolved in water and further purified by descending paper chromatography for 10 h using *N*-butanol/acetic acid/H_2O (5 : 1 : 4, by vol.) as a solvent system and 3 MM Whatman chromatography paper. The chromatogram was then stained with ninhydrin reagent containing cadmium acetate, and the corresponding fractions eluted with water and subsequently lyopholized.

Analysis of amino acids. In order to determine the presence of a peptide in the partially purified extract the sample was subjected to amino acid analysis before and after acid hydrolysis. To the dried sample was added 100 μl 6 N HCl, 0.25% phenol, 1% thioglycolic acid, after which the tube was evacuated and sealed. After 22 h at 110°C, the acid was removed in vacuo and the dried sample was dissolved in sodium citrate buffer (pH 2.2) and analyzed on a modified Beckman amino acid analyzer with a micro-bore column [15].

Amino acid sequence analysis. The Edman degradation procedure of Gray [16] was used to determine the amino acid sequence of the peptide. N-Terminal amino acids were, however, identified by the subtractive fashion. Samples were analyzed for amino acid composition after each step of Edman degradation, and the decrease in an amino acid was considered as the amino acid removed from the N-terminus.

Antigonadotropic assay. In each purification step the antigonadotropic activity of the extract was assayed by the ability of the extract to reduce compensatory ovarian hypertrophy in adult mice [11]. Charles River CD-1 female mice were utilized in the assays. The animals were received at 28 days of age, housed in a light-dark environment of 14-10 h at 25°C and used at either 1 or 2 weeks after receipt. The extract was dissolved in 0.9% NaCl and each animal received a single intraperitoneal injection of the appropriate dose of extract delivered in 0.1 ml diluent at the time of unilateral ovariectomy. The animals were killed 5 days later and the remaining ovary removed. The weight of this ovary was then compared with that of the contralateral ovary which had been removed earlier and calculated as percent hypertrophy. The mean percent compensatory ovarian hypertrophy was determined for each group and compared by Student's paired '*t*' test.

Results

Each of the fractions of the pineal extract obtained after ion-exchange chromatography was tested for antigonadotropic activity by administering 2.5

TABLE I

REDUCTION OF COMPENSATORY OVARIAN HYPERTROPHY BY A PINEAL EXTRACT PARTIALLY PURIFIED BY ION-EXCHANGE CHROMATOGRAPHY

Each animal was treated with 2.5 gequiv. of the appropriate fraction. n.s., not significant.

Fraction	Residue wt. (mg/gequiv.)	*n*	% compensatory ovarian hypertrophy ($\bar{x}$ ± S.E.)	*P* vs. control
Control	—	10	44.7 ± 4.3	—
H_2O	4.2	8	57.4 ± 1.9	<0.025
0.2 M pyridine (pH 4.0)	3.3	8	52.3 ± 5.9	n.s.
0.2 M pyridine (pH 5.0)	2.2	9	30.6 ± 4.6	<0.05
1 M pyridine (pH 8.5)	8.6	10	21.1 ± 5.7	<0.005

gequiv. (equivalent to wet weight of starting material) per animal per group (Table I). In the two experiments performed, the water fraction displayed activity which was stimulatory to ovarian weight. The material eluted with 1 M pyridine displayed more antigonadotropic activity than that observed with the 0.2 M pyridine (pH 5.0) eluant.

The 1 M fraction was further purified by gel filtration to yield four fractions. Each of these fractions was administered as 2.5, 5.0 and 10.0 gequiv. per animal and found to reduce compensatory ovarian hypertrophy (Table II). Fraction III was observed to be the most potent of the four and was further purified by electrophoresis.

The material from fraction III yielded 16 ninhydrin-positive areas on the paper electrophoretogram, some of which were not examined for antigonadotropic activity. In all instances each fraction was administered as 2.5 gequiv. with some fractions reexamined at the 5.0 gequiv. dose (Table III). Of these

TABLE II

ANTIGONADAL EFFECTS OF THE 1 M PYRIDINE ION-EXCHANGE FRACTION AFTER PARTIAL PURIFICATION BY GEL FILTRATION

n.s., not significant. COH, compensatory ovarian hypertrophy.

Fraction	Tube No.	Residue wt. (mg/gequiv.)	Protein content (μg/gequiv.)	Dose (gequiv.)	*n*	% COH ($\bar{x}$ ± S.E.)	*P* vs. control
Control				—	8	57.7 ± 8.1	
I	14—19	0.26	10.9	2.5	8	27.7 ± 5.5	<0.01
				5.0	7	47.0 ± 9.4	n.s.
				10.0	8	45.4 ± 5.4	n.s.
II	20—30	0.29	3.9	2.5	8	17.1 ± 5.1	<0.001
				5.0	6	38.7 ± 2.0	<0.05
				10.0	8	6.1 ± 4.6	<0.001
III	31—36	0.23	11.0	2.5	8	—6.7 ± 8.2	<0.001
				5.0	8	28.1 ± 7.6	<0.025
				10.0	7	13.2 ± 3.4	<0.001
IV	37—45	3.90	6.1	2.5	8	21.9 ± 8.0	<0.01
				5.0	7	48.5 ± 15.1	n.s.
				10.0	7	4.7 ± 10.4	<0.005

TABLE III

EFFECT ON COMPENSATORY OVARIAN HYPERTROPHY (COH) OF FRACTION 1 M-III AFTER PARTIAL PURIFICATION BY ELECTROPHORESIS

RL is the ratio of migration distance of ninhydrin-positive fraction and that of lysine from origin in cm. For COH not all fractions were tested for activity at the same time, but each study had appropriate control animals. Thus, the $\bar{x}$ % COH ± S.E. was calculated but the compiled results are reported as percent change from the respective control group. n.s., not significant; n.d., not determined.

Fraction	R_L	Dose (gequiv.)	COH (% change from control)	P
16	0.12—0.16	2.5	—71.5	<0.025
15	0.16—0.21	2.5	0	n.s.
14	0.21—0.24	2.5	—27.2	n.s.
13	0.24—0.34	2.5	+12.0	n.s.
12	0.34—0.38	2.5	—18.6	n.s.
11	0.38—0.41	2.5	—37.2	n.s.
10	0.41—0.46	2.5	n.d.	—
9	0.46—0.49	—	n.d.	—
8	0.54—0.58	—	n.d.	—
7	0.58—0.62	—	n.d.	—
6	0.62—0.66	2.5	—88.4	<0.01
		5.0	—87.8	<0.01
5	0.66—0.77	2.5	—84.3	<0.005
		5.0	—77.9	<0.025
4	0.77—0.83	2.5	—83.8	<0.001
		5.0	—48.5	<0.05
3	0.83—0.94	2.5	—59.1	n.s.
2	0.94—0.98	—	n.d.	—
1	0.98—1.21	—	n.d.	—

fractions tested, four were found to be inhibitory to compensatory ovarian hypertrophy. Three of the fractions (fractions 4—6) were grouped closely together (R_L 0.62—0.83) while the fourth active fraction (fraction 16) was some distance from these (R_L 0.12—0.16). Fractions 4—6 were observed to be active at the two dose levels utilized. Although fraction 3 reduced compensatory ovarian hypertrophy by 59.1% the variance was such that this was not statistically significant.

Inasmuch as fraction 5 was central to the three clustered fractions this frac-

TABLE IV

ANTIGONADOTROPIC ACTIVITY OF FRACTION 1M III-5 AFTER SEPARATION BY PAPER CHROMATOGRAPHY

Each fraction was administered as 2.5 gequiv. per animal. n.s., not significant. COH, compensatory ovarian hypertrophy.

Fraction	R_F	n	% COH ($\bar{x}$ ± S.E.)	P vs. control
Control		8	43.0 ± 6.3	—
A	0.130—0.206	8	23.9 ± 5.8	<0.05
B	0.207—0.257	8	21.9 ± 5.6	<0.025
C	0.258—0.297	8	34.3 ± 9.4	n.s.
D	0.298—0.363	8	49.9 ± 8.8	n.s.
E	0.364—0.409	8	23.8 ± 8.2	n.s.

TABLE V

AMINO ACID CONTENT OF FRACTION 5A AND 5B

Amino acid content is based on 118 gequiv. after acid hydrolysis. Control, blank chromatography paper.

Amino acid	Amino acid content (nM)		
	Control	5A	5B
Asp	1.3		2.9
Thr	0.5	19.2	4.7
Ser	0.2	21.2	8.8
Glu	0.2	—	2.2
Gly	2.7	—	4.3
Ala	1.4	—	3.6
Met	—	—	0.5
Pro	—	—	1.8
Val	—	—	0.0
Lys	0.5	23.3	—
Ile	—	—	0.3
Leu	—	—	1.1
Arg	—	—	2.6

tion was further purified by paper chromatography. Five fractions were eluted from the chromatogram, two of which were active (Table IV). Amino acid analysis of the two fractions indicated that fraction 5A was in pure form containing a peptide consisting of threonine, serine and lysine, while 5B was not in homogeneous form (Table V). Fraction 5A contained 0.538 nmol amino acids/gequiv. while 5B contained 0.782 nmol amino acids/gequiv. The results of the first and second cycles of subtractive Edman degradation of the tripeptide in fraction 5A indicated that the amino acid sequence of the peptide was threoninylserinyllysine.

Discussion

The biological parameter utilized in this study to determine the antigonadal properties as a guide for the purification of the peptide is related to the animal's ability to secrete sufficient gonadotropins. The mechanism of compensatory ovarian hypertrophy is thought to be dependent upon gonadotropins [17], particularly follicle-stimulating hormone [18]. Based on this phenomenon a number of laboratories have utilized this bioassay as a means to study the antigonadotropic properties of the pineal constituents [9,10].

The present study clearly demonstrates the presence of a small peptide in bovine pineal extract which possesses properties inhibitory to compensatory ovarian hypertrophy in mice. It would appear that in addition to the isolated tripeptide, other peptides are present which display antigonadal characteristics. Fraction 5B, while heterogeneous, probably contains a small peptide and demonstrates a higher degree of potency in reducing compensatory ovarian hypertrophy than the isolated tripeptide. Moreover, the fractions obtained after electrophoresis other than fraction 4, which possess antigonadal properties, may contain peptides.

Thus, throughout the purification scheme several fractions apparently con-

taining small pepetides were observed to exhibit antigonadal properties. Whether some of these may be residues from a larger molecule cannot be determined at this time. However, a number of peptides have been found in pineal tissue (see Ref. 10). Thieblot and Blaise [19] isolated a small peptide from bovine pineal tissue which possessed antigonadal properties. Bovine pineal extracts devoid of melatonin have been shown to possess antireproductive properties [20] with the partially purified material being more potent than melatonin in blocking compensatory ovarian hypertrophy [21]. Subsequent studies with this extract have shown that the material was rendered inactive after the addition of proteolytic enzymes [22] indicating the active material was a small peptide. Rosenblum et al. [23] have demonstrated that an antireproductive fraction from bovine pineal extracts contained a peptide separate from arginine vasotocin based on amino acid composition of this fraction. It would appear that the isolated tripeptide and the peptide in fraction 5B in the present study are different from that isolated by Rosenblum et al. [23].

In previous studies with bovine pineal extracts partially purified by gel filtration it was demonstrated that more than one fraction possessed antigonadotropic activity [11,12]. The active material was capable of suppressing ovulation and the proestrous surge of luteinizing hormone in rats [24]. The fraction also reduced serum concentrations of LH and testosterone in male rats [12]. Further studies indicated that the material responsible for this activity was a small basically charged peptide separate from arginine vasotocin [25]. Utilizing in vitro methods Ebels [26] demonstrated that various fractions of partially purified ovine pineal extracts acted on either the hypothalamus or anterior pituitary to suppress the relase of GnRH or gonadotropins, respectively. Ota et al. [27] found after partial purification of bovine pineal powder a substance of $M_r < 1000$ which reduced ovulation in mice. Thus, from these studies it is apparent that a number of substances (presumably peptides) are present in extracts of pineal tissue which possess characteristics inhibitory to reproductive hormones. In a preliminary study we have observed the tripeptide to inhibit serum FSH in unilaterally overiectomized mice and to inhibit the GnRH-induced release of FSH in male rats [28]. In the present study a number of fractions were observed to reduce compensatory ovarian hypertrophy, a phenomenon dependent upon sufficient plasma concentrations of gonadotropins. Whether these fractions, which appear to contain peptides, are similar to those in the afore-mentioned studies is difficult to determine. However, based on the amino acid contents, none of the antigonadal fractions (fractions 4—6 and 16) eluted after electrophoresis contain arginine vasotocin.

Inasmuch as the extract was subjected to cation-exchange chromatography and gel filtration it was felt that all the antigonadotropic material would be found quite distant from the origin of the electrophoretogram. While this was true for most, one antigonadal fraction (fraction 16) was found near the origin. In a preliminary study, we have observed, utilizing a different sequence of purification procedures for bovine pineals, a similar situation [25]. A similar occurrence has been observed with ovine pineal extracts [26].

A significant increase in compensatory ovarian hypertrophy was observed in the mice after administering the fraction eluted with water from the cation-exchange column. This would indicate that some substance(s) present in the

eluant was stimulatory to gonadotropin release. A stimulatory factor has been reported in ovine pineal extract [29] and was found to be accumulated by chromatography on a weak cation-exchange column [30]. Subsequent studies indicate the compound responsible for this activity is $500 < M_r < 1000$ [26]. Whether the material in the present study is similar to that mentioned above is not possible to determine but does provide additional evidence for a stimulatory factor present in pineal tissue.

In conclusion, bovine pineal tissue appears to contain a number of substances other than melatonin and arginine vasotocin which modify reproductive function. One of these antigonadal substances was determined to be a tripeptide. Other fractions, apparently containing peptides, were also inhibitory to compensatory ovarian hypertrophy, while one crude fraction was stimulatory. Inasmuch as compensatory ovarian hypertrophy is dependent upon gonadotropins, it is felt that the activity of these antigonadal fractions either reduce the release or gonadal uptake of gonadotropins.

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