STUDIES ON THE PRESENCE OF VASOPRESSIN, OXYTOCIN AND VASOTOCIN IN THE PINEAL GLAND, SUBCOMMISSURAL ORGAN AND FETAL PITUITARY GLAND: FAILURE TO DEMONSTRATE VASOTOCIN IN MAMMALS

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SUMMARY

The demonstration of vasotocin in the mammalian pineal gland, subcommissural organ and fetal pituitary gland by bioassay has led to hypotheses regarding the function of this hormone in various reproductive processes.

Preliminary examinations of the pineal gland and subcommissural organ with a specific radioimmunoassay failed to show vasotocin immunoreactivity. The presence of vasotocin, vasopressin and oxytocin in the pineal gland, subcommissural organ and fetal neurohypophysis was therefore investigated, using three specific radioimmunoassays. Frog and chicken pituitary glands were used to validate the vasotocin radioimmunoassay.

Direct measurements in diluted homogenates of pituitary glands from frogs, chickens, mid-term fetal sheep and near-term fetal seals revealed the presence of vasotocin only in the frog and chicken pituitary glands, while vasopressin and oxytocin were found in the two fetal pituitary homogenates.

Vasopressin and oxytocin were measured in homogenates of rat and bovine pineal glands and in preparations of the subcommissural organ of rats and rabbits after extraction with Vycor glass powder, but no specific vasotocin immunoreactivity was observed. These results indicate a discrepancy between the reported biological activity of vasotocin in the pineal gland, subcommissural organ and fetal pituitary gland and the immunoreactivity of this material, which can at present only be explained by the presence of a peptide which is structurally closely related to, but not identical with, vasotocin.

INTRODUCTION

Arginine-vasotocin (AVT) is generally considered to be the ancestral peptide of the neurohypophysial hormones, arginine-vasopressin (AVP) and oxytocin, in mammals. It has been assumed that the presence of AVT was limited to the hypothalamo-neurohypophysial system of lower vertebrates (Acher, 1974). However, Pavel (1965) reported the presence of AVT in the pineal gland and Vizsolyi & Perks (1969) in the neurohypophysis of mid-term fetal sheep and seals. It was thought that AVT was the antgonadotrophic factor from the pineal gland (Pavel & Petrescu, 1966; Benson, 1977). In addition, a function for this hormone in the maintenance of intra-uterine water balance of the fetus has been proposed by Vizsolyi & Perks (1976).

Vasotocin was demonstrated in these studies by bioassay. Amino-acid analysis confirmed its presence in the bovine pineal gland (Cheesman, 1970) and in the fetal seal pituitary gland

(Perks & Vizsolyi, 1973). However, a specific radioimmunoassay of this hormone has not yet been reported. Vasopressin antisera have been used to measure AVT in the rat pineal gland, rabbit subcommissural organ (SCO; Rosenbloom & Fisher, 1975) and in fetal pituitary glands of man and sheep (Skowsky & Fisher, 1977), while Legros, Louis, Demoulin & Franchimont (1976) used an oxytocin antiserum to measure AVT in the human fetal pineal gland.

When we repeated our previous AVT measurements, which were also performed with an AVP antiserum in a preliminary study (Swaab, Van Leeuwen, Dogterom & Honnebier, 1977), with an antiserum raised against AVT itself, the presence of AVT in fetal rat pituitary glands could not be confirmed (Swaab, Boer, Boer, Dogterom, Van Leeuwen & Visser, 1978). Also, the pineal gland and SCO showed no specific AVT immunoreactivity (Pévet, Dogterom, Buijs, Swaab & Janssens, 1978). The present report describes the determination of AVP, AVT and oxytocin in the pituitary glands of 7-day-old chickens, adult frogs, near-term fetal seals, mid-term fetal sheep, in the pineal glands of cattle and rats (Wistar and Brattleboro strain), in commercially available SCO of rabbits, and in brain samples containing the SCO of rats (Wistar and Brattleboro strain).

MATERIALS AND METHODS

All peptides used in the present study were synthetic products generously supplied by Dr H. M. Greven (Organon, Oss, The Netherlands), except mesotocin which was synthesized and generously given by Dr F. Vandesande, University of Ghent, Belgium. The following preparations were used: AVP (for immunization: 180 i.u./mg; for standard curves and labelled hormone: 509 i.u./mg); AVT (234 i.u./mg); oxytocin (509 i.u./mg). The biological activities of AVP, AVT and oxytocin were determined by the manufacturer (pressor activity in the rat for AVP and AVT; depressor activity of oxytocin in the cockerel). The peptides were stored in stock solutions of suitable concentration in 0.25% acetic acid at -20°C .

Radioimmunoassay

The procedure for raising antibodies, preparation and purification of labelled hormones, preparations of the standard curves and extraction of the hormones from the samples with activated Vycor glass powder have been described for the radioimmunoassay of AVP and oxytocin (Dogterom, Van Wimersma Greidanus & Swaab, 1977; Dogterom, Van Wimersma Greidanus & De Wied, 1978). To produce AVT antiserum AVT itself was coupled to thyroglobulin using carbodiimide (Skowsky & Fisher, 1973) and the conjugate was injected subcutaneously and intramuscularly into White New Zealand rabbits at intervals of 2 weeks. The radioimmunoassay of AVT was similar to that of oxytocin. To control the specificity of the radioimmunoassays and the recovery of the extraction procedure, standard amounts of AVP, oxytocin or AVT were added to tubes containing the same solutions in which tissue samples were sonicated. Six tubes were routinely included in each extraction experiment each containing the following medium: 1.0 ml 0.1 M-HCl; 100 μl 1.0 M-NaOH; 0.5 ml barbitone buffer (pH 7.0); two of these tubes also contained 128 pg AVT, 128 pg oxytocin and 32 pg AVP; two contained 32 pg AVT, 32 pg oxytocin and 8 pg AVP; two contained no hormone. pH Values of all tubes were checked and adjusted to between 6 and 7. The detection limit for the AVP and AVT assay was 1 pg/sample and for the oxytocin assay it was 2 pg/sample.

Sample preparation

Frog and chicken

Frogs (*Rana esculanta*) and 7-day-old chickens (*Gallus domesticus*) were decapitated between 10.00 and 11.00 h; the pituitary glands were removed immediately and put in 1 ml ice-cooled 0.1 M-HCl. The tissue was then sonicated for 1 min at 20 Hz and stored at

–70 °C. Hormone measurements were performed directly in serial dilutions in barbitone buffer immediately before use. The dilutions used varied depending upon which hormone was being measured (see Results). Each dilution was measured in triplicate in each assay.

Seal and sheep fetuses

Pituitary glands from five sheep fetuses at 72 days of gestation (two twins of both sexes and one female) were removed from the fetal skull immediately after killing the three pregnant ewes (*Ovis aries*). The glands were put in 250 µl 0.1 M-HCl, sonicated and stored at –20 °C until serial dilutions were made in barbitone buffer.

Lyophilized pituitary glands of near-term fetal seals (*Callorhinus ursinus*) were kindly donated by Professor A. M. Perks and Dr E. Vizsolyi, Vancouver, Canada. This material had been stored for approximately 10 years. The pituitary glands were weighed, sonicated in 1 ml 0.1 M-HCl and stored at –20 °C. Dilutions of the fetal seal and sheep pituitary homogenates were measured in triplicate in each assay.

Rat samples

Male rats of an inbred Wistar strain (CPB-TNO, Zeist, The Netherlands) weighing 150–200 g, or male rats of the Brattleboro strain (homozygous for hereditary diabetes insipidus: homo-DI from CPB-TNO) of the same weight were used. The animals had free access to water and food. The animal house was illuminated from 07.00 to 19.00 h. Rats were anaesthetized with pentobarbitone (6 mg/100 g body weight, i.p.) and perfused intracardially with 0.9% saline before decapitation to remove the blood from the tissue; the pineal glands and SCO were then removed and kept on a disk in melting ice. The latter samples consisted of a microscopically dissected piece of tissue containing the SCO and surrounding brain tissue. The tissues were weighed and then sonicated in 0.5 ml 0.1 M-HCl and stored at –70 °C.

Pineals of 19-day-old rat fetuses and of 1-day-old rat pups were isolated immediately after decapitation and also sonicated in 0.5 ml 0.1 M-HCl and stored at –70 °C. All the samples were neutralized with 50 µl 1 M-NaOH and 1.5 ml barbitone buffer (pH 7.0) was added immediately before the extraction procedure. Each extract was measured in duplicate in each assay.

Rabbit and bovine samples

The SCO preparations of rabbits and pineal glands of cattle were obtained from Pelfreeze Biological Inc. (Rogers, Arkansas, U.S.A.). The tissue arrived in solid CO₂ and was stored at –70 °C until homogenized and extracted as described for the rat samples.

Calculation and statistics

Calculations were performed with a computer using a Fortran IV program. The best-fit linear regression of the standard curves was calculated using a logit-log transformation (Rodbard & Lawald, 1970; B , percentage binding observed; B_0 , percentage of initial binding). The same procedure was used to calculate the regression lines of the serial dilutions of the pituitary measurements. Differences between coefficients of regression of dilution curves and standard curves were tested with Student's t -test, considering $P < 0.05$ as significant. All results are given as means $\pm$ S.E.M. The data were corrected for recovery.

RESULTS

Radioimmunoassay for AVT

The immunization procedure employed resulted in several AVT antisera with a maximal binding capacity of approximately 80% of a small amount of AVT tracer. The AVT tracers had specific activities varying between 250 and 500 Ci/g. The AVT antiserum code 5 (22–6)

appeared to be highly sensitive and specific; the cross-reactivity with mesotocin was <0.1%, while the cross-reactivity with oxytocin and AVP decreased rapidly with increasing levels of AVT (Fig. 1). The recovery of AVT from either barbitone buffer or homo-DI rat plasma after the extraction procedure with activated Vycor glass powder was $49.5 \pm 4.5\%$ ($n = 25$) over a range of 4–128 pg standard AVT.

Fig. 1. Representative standard curve of vasotocin (AVT; ○) with AVT antiserum (code 5, 22–6; dilution 1 : 20 000) and cross-reaction curves with closely related peptides: mesotocin (Δ); oxytocin (×); vasopressin (□) (each point represents the mean of three values). B , % binding observed; B_0 , % initial binding.

Levels of AVT in homo-DI rat plasma were not detectable and it thus served as a control for non-specific values. In blank tubes containing HCl, NaOH and the barbitone buffer, non-specific values were not found for any of the three assays. The detection limit of the radioimmunoassay for AVT was 1 pg/sample. Within-assay variability for the AVT assay was 19.3% (eight identical samples containing 16 pg AVT in one assay in duplicate) and between-assay variability was 19.6% (eight assays in duplicate of identical samples containing 16 pg standard AVT).

Assay results

The measurements of AVP, oxytocin and AVT in serial dilutions of homogenates of pituitary glands of fetal seals, fetal sheep and frogs made it possible to calculate regression coefficients of the dilution curves and to control for parallelism with standard curves. Figure 2a shows examples of dilution curves of AVP measurements in pituitary homogenates compared with a standard curve. Note that the immunoreactivity of AVP in the pituitary glands of fetal seals and sheep is parallel with the standard curve, while no AVP immunoreactivity was measured in the frog pituitary gland at the lowest dilution used (1 : 2000). Figure 2b shows examples of curves of oxytocin measurements in dilutions of pituitary homogenates of the same three species. The difference between the regression coefficient of standard curves and dilution curves of the oxytocin measurements in frog pituitary tissue was not significant ($P > 0.05$), while regression coefficients of the curves of the fetal material were the same as the standard curves. The regression coefficient of the dilution curves of frog pituitary tissue was similar to this value of the cross-reaction curve of

Fig. 2. Representative standard curves (○) of (a) arginine-vasopressin, (b) oxytocin and (c) arginine-vasotocin and representative dilution curves of pituitary homogenates of frog, fetal seal and fetal sheep. The lowest dilution used is indicated for each species in each assay. Each point of the curves represents the mean of three determinations. B , % binding observed, B_0 , % initial binding.

mesotocin (regression coefficient of mesotocin = -1.49 ± 0.09) in the oxytocin radio-immunoassay, which points to cross-reaction of mesotocin (Table 1). Figure 2c shows examples of the AVT regression lines of the serial dilutions of the pituitary material and of a standard curve. No immunoreactivity was observed in fetal sheep pituitary glands at the 1 : 500 dilution, while significant ($P < 0.05$) non-parallel immunoreactivity was observed in fetal seal pituitary tissue. Frog pituitary glands, on the other hand, showed parallel dilution curves in the AVT assay. The coefficient of regression of fetal seal pituitary glands was close to the value of the cross-reaction curve of AVP in the AVT assay (regression coefficient of AVP = -0.82 ± 0.11), indicating cross-reaction of AVP in the AVT radio-immunoassay (Table 1).

Table 1. *Regression coefficients of standard curves for arginine-vasopressin (AVP), oxytocin and arginine-vasotocin (AVT) and of dilution curves of homogenates of HCl-extracts of pituitary glands from frog, fetal seals (near-term) and fetal sheep (at 72 days of gestation). (Values are means $\pm$ S.E.M.; regression coefficients of the number of dilution and standard curves are shown in parentheses)*

	AVP	AVT	Oxytocin
Synthetic standard	-2.25 ± 0.22 (9)	-2.17 ± 0.16 (8)	-2.22 ± 0.16 (10)
Frog	—	-2.75 ± 0.31 (5)	-1.51 ± 0.06^{NS} (3)
Fetal seal	-2.33 ± 0.11 (5)	$-1.06 \pm 0.15^*$ (5)	-2.17 ± 0.13 (5)
Fetal sheep	-2.03 ± 0.25 (5)	—	-2.27 ± 0.05 (5)

NS, not significant and * $P < 0.05$ compared with appropriate synthetic standard (Student's *t*-test).

Table 2 summarizes the pituitary hormone content and the AVP : oxytocin ratio of the pituitary glands. The pituitary glands of 7-day-old chickens contained $1.19 \pm 0.18 \mu\text{g}$ AVT/gland ($n = 5$).

Table 2. *Neurohypophysial hormones (μg /hypophysis) in pituitary glands of frogs, fetal sheep (at 72 days of gestation) and fetal seals (near-term). (Values are means $\pm$ S.E.M.; number of animals in parentheses)*

Pituitary gland	AVT	Oxytocin	AVP	AVP : oxytocin
Frog	0.51 ± 0.10 (4)	non-parallel	undetectable	—
Fetal seal	non-parallel	7.0 ± 0.7 (5)	32.1 ± 3.0 (5)	4.6
Fetal sheep	undetectable	0.06 ± 0.01 (5)	0.74 ± 0.18 (5)	12.3

Table 3 summarizes the results of hormone measurements after extraction of homogenates of pineal glands and preparations of SCO. Oxytocin was present in all samples and in the rabbit SCO, even in nanogram quantities. Vasopressin was also detected in all the samples except those from the homo-DI rats. Vasotocin was observed only in the rabbit SCO, but this AVT immunoreactivity can be fully explained by cross-reaction with the high amounts of AVP present in this preparation. The AVT was also not detectable in the pineals of 19-day-old rat fetuses and 1-day-old rat pups.

Table 3. Mean ($\pm$ S.E.M.) neurohypophyseal hormone concentration (pg/mg wet tissue) of rat and bovine pineal glands and of rat and rabbit subcommissural organs with surrounding tissue (SCO). Measurements of each hormone were performed in three samples each containing three (rat) or one (bovine) pineal or one SCO. The detection limits of the assays were 1 pg/sample for vasopressin (AVP) and vasotocin (AVT) and 2 pg/sample for oxytocin

Tissue	AVT	Oxytocin	AVP
Rabbit SCO*	3.2 $\pm$ 0.9	1080 $\pm$ 240	3320 $\pm$ 520
Wistar rat SCO	undetectable	16.8 $\pm$ 1.6	28.0 $\pm$ 5.0
Brattleboro rat SCO	undetectable	13.2 $\pm$ 2.0	undetectable
Wistar rat pineal	undetectable	1.3 $\pm$ 0.8	2.7 $\pm$ 0.8
Brattleboro rat pineal	undetectable	8.7 $\pm$ 3.7	undetectable
Bovine pineal	undetectable	3.7 $\pm$ 1.7	3.7 $\pm$ 0.7

* Supplied by Pelfreeze.

DISCUSSION

In addition to the previously described radioimmunoassays for AVP and oxytocin (Dogterom *et al.* 1977, 1978) the present paper describes a radioimmunoassay for AVT using an antiserum that was raised against AVT itself. This assay has similar features to the AVP and oxytocin assays with regard to the binding capacity of the antiserum used, the specific activity of labelled hormone, the sensitivity of the standard curve and recovery in the extraction procedure. The three antisera employed in the present study all had a specificity that discriminated between and measured all three hormones in the same sample. The specificity of the AVT assay was further indicated by the finding that two other synthetic AVT preparations (obtained from Bachem. Inc. and from Dr Vandesande, Ghent, Belgium) also gave parallel standard curves in the present assay, though with lower sensitivity (probably because the preparations were less pure). The non-parallel immunoreactivity of near-term fetal seal pituitary glands in the AVT radioimmunoassay was equivalent to 450 pg/gland based on the lowest dilution employed (1 : 25). This amount was extremely low compared with the AVP content of 32.1 μ g/gland and can be fully explained by cross-reaction of AVP in the AVT assay. The AVP : oxytocin ratio of the near-term fetal seal pituitary glands in the present experiments was higher than the value reported by Perks (1977) and appeared to be more typical of mid-term fetal pituitary glands. No evidence for the presence of AVT in near-term fetal seal pituitary glands, was obtained in our assay system; however, analysis of the biological activities of this near-term seal tissue does not suggest the presence of AVT at this late stage of development (E. Vizsolyi & A. M. Perks, personal communication). Evidence for the presence of AVT in mid-term fetal sheep was lacking, while the AVP : oxytocin ratio for this material is in good agreement with the value reported by Vizsolyi & Perks (1976). The activity of frog bladder induced by fetal pituitary glands might be due to AVT-like compounds which are different from AVT itself and which do not cross-react in the AVP and oxytocin assays. It is quite possible that unknown peptides which resemble AVT are present in the hypothalamo-neurohypophyseal system of fetal mammals. Recently, at least four low molecular weight peptides which hitherto had not been found and which still remain to be characterized have been isolated from the supraoptic nucleus of the adult rat (Gainer, Sarne & Brownstein, 1977). The existence of such compounds during ontogeny has yet to be investigated. A similar discrepancy between biological activity and immunoreactivity is present in adult rat and bovine pineal glands and in rat and rabbit SCO because of the lack of any specific AVT immunoreactivity. Whereas oxytocin was found in all samples investigated and AVP in the SCO of Wistar rats and rabbits and in the pineal glands of cattle and rats, low AVT immunoreactivity was found only in the SCO of rabbits. This immunoreactivity should be interpreted as cross-reactivity with AVP. Immunocytochemical studies have revealed that numerous AVP and oxytocin-

containing fibres are present in brain tissue immediately adjacent to the SCO (Weindl & Sofroniew, 1978; Buijs & Pévet, 1980), the SCO, the pineal stalk and the anterior part of the pineal (Buijs & Pévet, 1980). Immunocytochemistry on the pineal gland of the rat revealed that only one of four potent AVT antisera gave positive results. This indicates that the antiserum did not stain AVT itself but rather an AVT-like compound (Pévet, Dogterom, Buijs, Ebels, Swaab & Arimura, 1980). Neacşu (1972) reported the isolation from bovine pineal glands of a 14 amino-acid peptide with AVT-like biological activity. In conclusion it may be said that in our radioimmunoassay system, any evidence for the presence of AVT in mammals is absent. Nevertheless, levels below the detection limit of the AVT radioimmunoassay could be present in the samples studied but according to the literature such levels would have been sufficient for detection. The present findings illustrate that further characterization of peptides from mammalian fetal hypothalamic or neurohypophyseal material and adult pineal glands is necessary to elucidate the present discrepancies between bioassay, immunocytochemistry and radioimmunoassay measurements.

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