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THE INTERRELATIONSHIPS BETWEEN NONAPEPTIDE AND STEROID HORMONES SECRETION BY BOVINE GRANULOSA CELLS *IN VITRO*

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Summary—The effects of adding oxytocin (OT) and arginine-vasopressin (AVP) on progesterone and estradiol-17β secretion by bovine granulosa cells in culture were studied. The influence of these steroids on OT and AVP release was also evaluated. OT (1, 10, 100 or 1000 mIU/ml) stimulates both progesterone and estradiol output. Small doses (10 pM/ml) of exogenous progesterone or estradiol stimulated a surge in OT, while the intermediate doses (100 or 1000 pM/ml) had no influence, and large doses (10,000 pM/ml) inhibited OT secretion by granulosa cells. Thus, a potential regulatory loop between OT and steroid hormone release by granulosa cells was demonstrated. Stimulation of a surge in steroids by OT, activation of OT release by small doses of steroids and inhibition of OT secretion by excess steroids may suggest the existence of a feedback mechanism regulating these hormones production. Addition of AVP (1, 10, 100 or 1000 pM/ml) inhibited progesterone and stimulated a surge in estradiol, while steroid hormones did not induce AVP release. These data suggest the regulation of ovarian steroidogenesis by AVP, feedback influences are less likely.

INTRODUCTION

There is considerable evidence that nonapeptide hormones—oxytocin (OT) and arginine-vasopressin (AVP)—can be synthesized within the mammalian gonads and may be involved in the autocrine regulation of cAMP, prostaglandins, steroid hormone production and luteinization of ovarine cells [for reviews see 1–3]. But the interrelationships between ovarian nonapeptides and steroid hormone secretions are still not clear. In particular, there is practically no evidence for steroid action on gonadal nonapeptide hormone secretion. Estradiol treatments enhanced blood AVP levels in rats [4] and 5α-androst-16-en-3-one increased OT concentrations in porcine blood [5]. However, this information is difficult to interpret since blood nonapeptides may be of both hypothalamic and gonadal origin. In *in vitro* experiments neither estradiol [6] nor the steroidogenesis inhibitor aminoglutethimide [7] had any influence on OT production by bovine luteal and granulosa cells in culture, i.e. an influence of steroids of ovarian nonapeptide production was not proven.

On the other hand, investigations reporting the effects of nonapeptide hormones on ovarian steroidogenesis were performed mainly on luteal cells only, with contradictory results. In particular, according to Wuttke and coworkers [8–11], OT inhibited progesterone and 4-androstene-3,17-dione and stimulated estradiol secretion in porcine luteal cells in culture. The data obtained on luteal cells of other species did not support these observations: OT had no effect on progesterone production in human [12, 13] or cow [14, 15] luteal cells or even stimulated this process in human luteocytes [16]. Tan and coworkers [17–19] had noted a biphasic, dose-dependent pattern of OT effect (low OT doses can stimulate and higher ones inhibit progesterone secretion in human and bovine luteal cells).

There is little information about the influence of nonapeptide hormones other than OT on luteal cells. Pitzel and coworkers [8, 9] reported that AVP and lysine-vasopressin induced inhibition of progesterone and 4-androstene-3,17-dione (but not of estradiol) release from porcine luteal cells in culture. Arginine-8-vasotocin (AVT), a nonapeptide hormone close to OT or AVP [20], produced by the hypothalamus of fetal or newborn mammals [21, 22] and, according to some reports [23], also by brain ependyma

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and/or epihysis, can inhibit *in vitro* progesterone secretion by human [24] and porcine [8, 25] luteal cells. In the last culture AVT also suppressed estradiol (but not testosterone) secretion [25].

There is even less information about the involvement of nonapeptide hormones in the regulation of *follicular* ovarian cell steroidogenesis. In isolated mouse ovaries and bovine ovarian follicles AVT activated progesterone, inhibited estradiol, and had no marked influence on testosterone secretion [26, 27]. To clarify the interrelationships between follicular nonapeptides and steriod hormone secretion we conducted *in vitro* studies of (1) the influence of OT and AVP on progesterone and estradiol release, and (2) the actions of these steroids treatments on OT and AVP surges in bovine granulosa cells in culture.

EXPERIMENTAL

Hormones and chemicals

The following chemicals were used in this study: the medium TC-199 (Sigma, St Louis, MO); sodium pyruvate; calcium lactate; HEPES (Serva, Heidelberg, Germany); gentamicin (Pharmachim, Sofia, Bulgaria); zinc-insulin (Leciva, Prague, CSFR) and bovine fetal serum (Institute of Veterinary Medicine, Brno, CSFR). The following hormonal preparations were added: porcine FSH (Leciva, Prague, CSFR, Lot. 0705 88); synthetic OT (Gedeon Richter, Budapest, Hungary, Lot. 0250389); synthetic AVP (Institute of Organic Biochemistry, Prague, CSFR); progesterone (Calbiochem-Behring, La Jolla, CA, Lot. 3090066) and estradiol-17β (Serva). OT doses are presented in units as defined by the manufacturer 1 IU = 19.6 nM. All hormones were of research grade and dissolved in the incubation medium immediately before the experiment.

Preparation and incubation of granulosa cells

Ovaries at the late luteal, early and middle follicular phase of the estrous cycle were obtained from black–white cows 2–4 years old without visible reproductive abnormalities, killed at a local slaughterhouse and transported to the laboratory in moist gauze. Within approx. 1 h of removal ovaries were washed three times in sterile Krebs-Ringer solution. Thereafter the content of 2–5 mm follicles was aseptically aspirated using a 2 ml syringe with a 18 gauge needle. Opaque or hemorrhagic follicles were excluded. The content of syringes was transferred in non-conical centrifuge tubes. Granulosa cells were separated from follicular fluid by centrifugation at 200 ***g*** for 5 min. After removal of the supernatant the granulosa cells were resuspended in sterile TCM-199 supplemented by 5% bovine fetal serum. This operation (i.e. centrifugation, medium replacement and pipetting) was repeated three times. After the last centrifugation granulosa cells were resuspended in the incubation medium TCM-199, supplemented by 5% bovine fetal serum, 10 mIU/ml pFSH, 10 mIU/ml insulin, 6 mg/ml Hepes buffer, 200 mg/ml Na pyruvate, 600 mg/ml Ca lactate and 50 mg/ml antibiotic gentamicin. Cell viability as determined by Trypan blue stain was 75–90%. The cells were plated by adding 4×10^6 cells in 2 ml to Cell-Cult plate wells (Sterilin Ltd, Feltham, England). Primary cultures were incubated for 4 days at 37.5°C in 5% CO_2 in humidified air. After 2 days of culture the medium was replaced with the same medium in the presence or absence of various doses of nonapeptide or steroid hormones. At the end of the culture period the incubation medium was aspirated by a syringe and stored frozen at −40°C for the analysis of hormones.

Hormone radioimmunoassays (RIA)

The concentration of nonapeptides and steroid hormones were determined by RIA.

OT. The levels of OT were measured by commercial kits from the Institute for Research, Production and Application of Radioisotopes (UVVVP, Prague, CSFR) according to the manufacturer's protocol. Sensitivity of RIA was 1.2 pM/ml. Antiserum to OT cross-reacted <0.005% with AVP; 0.04% with lysine-vasopressin, 17% with AVT and 22.6% with desamine-OT. FSH and insulin displayed <0.001%. Interassay coefficients of variation varied between 11 and 12%, the intraassay coefficient of variation was 9%.

Vasopressin. Concentrations of this hormone were determined by RIA kits for AVP from the same firm. Sensitivity of the assay was 1.2 pM/ml. The cross-reaction of antiserum with lysine-8-vasopressin was 67.6%, with OT 0.025%, with AVT, FSH and insulin 0.0025%. The interassay coefficient of variation varied between 4 and 8%, the intraassay coefficient was 7%.

Table 1. Hormone content in the medium after 2 days of culture without and with bovine granulosa cells

	Hormone concentrations			
Culture	OT	AVP	Progesterone	17β-Estradiol
Without cells	0	0	94.5 ± 27.2	1.75 ± 0.12
With cells	51.6 ± 7.2	31.8 ± 9.3	301.1 ± 38.4	14.53 ± 1.53

××× Significant/$P < 0.001$/differences between the groups.

Progesterone. Progesterone levels were measured by commercial kits from the Institute of Radioecology and Nuclear Technics Application (URVJT, Košice, CSFR) according to their instructions. Sensitivity of RIA was 2 pM/ml. Antiserum to progesterone cross-reacted 58.6% with 11α-hydroxyprogesterone, 0.20% with corticosterone, and < 0.01% with cortisol, testosterone, estradiol-17β, estrone, 4-androstene-3,17-dione, FSH and insulin. Inter- and intraassay coefficients of variation did not exceed 17 and 9%, respectively.

Estradiol. Estradiol concentrations were determined by commercial kits of the Institute for Radioecology and Application of Nuclear Technics (URVJT) according to their instructions. Sensitivity of determination was 2.5 pM/ml. Cross-reaction of antiserum used with estrone was 25%, with estriol 1.84%, and with 20-α-OH progesterone, 4-androstene-3,17-dione, testosterone, cortisol and cortisol <0.001%. Inter- and intraassay coefficient of variation did not exceed 6 and 4%, respectively.

Statistics

Each experimental group was represented by four culture wells. Each experiment was repeated three times. The summarized results of all experiments are presented. Rates of hormone production were calculated on the basis of cells and substance concentrations to 10^6 viable cells/day. Significant differences between the groups were evaluated using the ANOVA test.

RESULTS

In preliminary experiments it was observed, that the medium, incubated for 2 days without the cells, contained no OT, AVP and relatively small amounts of progesterone and estradiol. On the other hand, a significant ($P < 0.001$) accumulation of all these hormones was observed after the medium was incubated with bovine granulosa cells (Table 1), indicating the production of OT, vasopressin, progesterone and estradiol by granulosa cells in culture.

In further experiments the stimulating influence of nonapeptide hormones—OT and AVP—on steroid hormone secretion by granulosa cells was demonstrated. In particular 1, 10, 100 or 1000 mIU/ml of OT significantly increased progesterone release ($P < 0.01$, <0.001, not significant and <0.001, respectively) (Fig. 1). Estradiol secretion was also enhanced after the same OT additions ($P < 0.001$, <0.05, <0.05 and <0.01, respectively) (Fig. 2).

In contrast to OT, AVP treatments (1, 10, 100 or 1000 pM/ml) were followed by a significant reduction of granulosa progesterone release ($P < 0.001$ in all cases) (Fig. 3). Estradiol

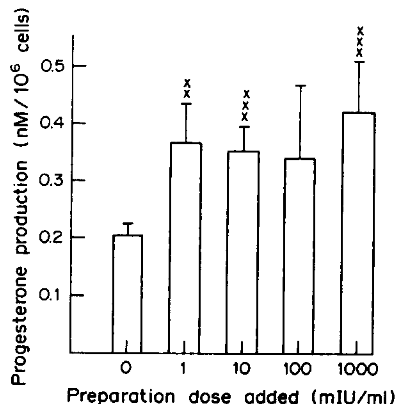

Fig. 1. Effect of OT on progesterone secretion by bovine granulosa cells *in vitro*. 1 IU of OT = 19.6 nM. Data are mean ± SEM. × ×, $P < 0.001$, × × ×, $P < 0.001$ compared with control (without treatment).

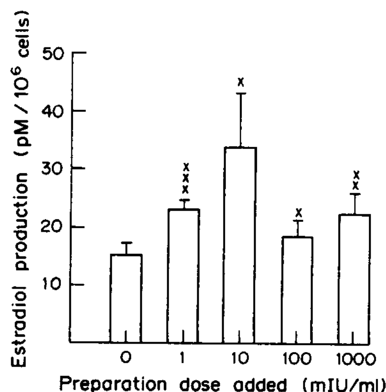

Fig. 2. Effect of OT on estradiol-17β secretion by bovine granulosa cells *in vitro*. 1 IU of OT = 19.6 nM. Data are means ± SEM. ×, $P < 0.05$, × ×, $P < 0.01$, × × ×, $P < 0.001$ compared with control (without treatment).

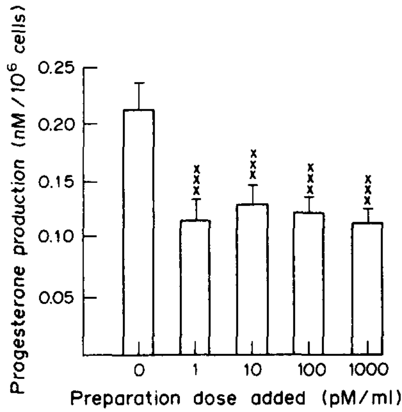

Fig. 3. Effect of AVP on progesterone secretion by bovine granulosa cells *in vitro*. Data are mean ± SEM. × × ×, $P < 0.001$ compared with control (without treatment).

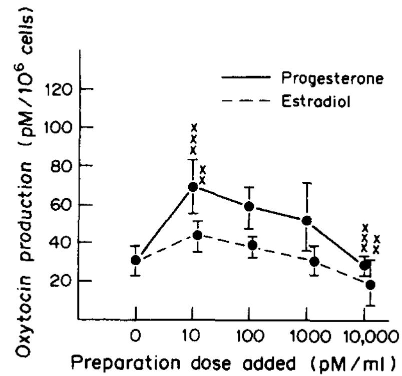

Fig. 5. Effect of progesterone (——) and estradiol-17β (- - -) on OT secretion by bovine granulosa cells *in vitro*. Data are mean ± SEM. × ×, $P < 0.01$, × × ×, $P < 0.001$ compared with control (without treatment).

secretion after these AVP additions was significantly increased ($P < 0.05$, < 0.001, < 0.05 and < 0.05, respectively) (Fig. 4).

In the course of the third series of experiments, the effects of steroid additions on granulosa nonapeptide hormone release were observed in some cases. In particular, small progesterone and estradiol doses (10 pM/ml) stimulated a surge in OT ($P < 0.001$ for progesterone and < 0.01 for estradiol). Greater steroid doses (100 or 1000 pM/ml) had no marked effect on this process. The greatest progesterone and estradiol doses (10,000 pM/ml) significantly inhibited OT release ($P < 0.001$ and < 0.01, respectively) (Fig. 5). All estradiol doses tested failed to influence AVP secretion. The highest progesterone doses used (10, 100 or 10,000 pM/ml) also had no effect on AVP production, although addition of 1000 pM/ml progesterone led to a slight, but statistically significant ($P < 0.05$) increase in AVP release (Fig. 6).

DISCUSSION

The augmentation of OT, AVP, progesterone and estradiol in the bovine granulosa cells incubation medium (Table 1) suggests that the cell culture used is viable and capable of producing both nonapeptide and steroid hormones. These observations support the available data about nonapeptide [1, 3] and steroid [28] hormone production by mammalian ovaries *in vivo* and *in vitro*.

The results presented in Figs 1 and 2 suggest a stimulatory influence of OT on both progesterone and estradiol secretion by bovine granulosa cells in culture. These observations are in agreement with those of Pitzel and coworkers [8, 10] and Jarry *et al.* [11], about OT-stimulated estradiol release by porcine luteal cells. Our results also support those of Tan and coworkers [17, 18] and Bennegard *et al.* [16], about the ability of OT to stimulate progesterone secretion in bovine and human

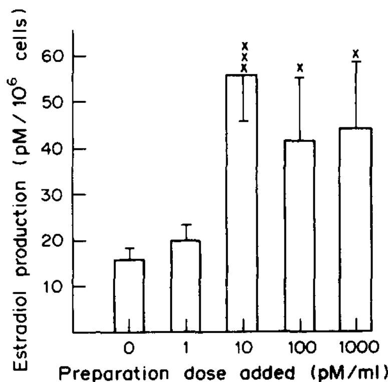

Fig. 4. Effect of AVP on estradiol-17β secretion by bovine granulosa cells *in vitro*. Data are mean ± SEM. ×, $P < 0.05$, × × ×, $P < 0.001$ compared with control (without treatment).

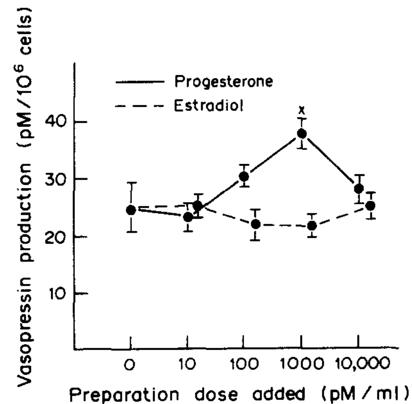

Fig. 6. Effect of progesterone (——) and estradiol-17β (- - -) on AVP secretion by bovine granulosa cells *in vitro*. Data are means ± SEM. ×, $P < 0.05$ compared with control (without treatment).

luteal cell cultures. However our observations do not confirm other reports that OT inhibits progesterone secretion in porcine luteal cells [8–11, 25] or has no effect on progesterone production in human [12, 13], bovine [1] and rat [14, 15] luteal cells *in vitro*. Our data provides the first evidence that OT can stimulate steroidogenesis not only in luteal but also in ovarian follicular cells.

In contrast to the effect of OT on steroid hormones, steroid influences on ovarian OT production have still not been described. Luck *et al.* [7] failed to find any influence of a steroidogenesis inhibitor on OT surge by bovine granulosa cells. Grazul *et al.* [6] observed no estradiol effect on OT production by bovine luteal cells.

In our experiments (Fig. 5) small doses of progesterone and estradiol stimulated, and high doses of inhibited OT release by bovine granulosa cells in culture. Such a biphasic effect may suggest the existence of a feedback mechanism regulating ovarian OT and steroid hormone production.

It may be hypothesized that in normal conditions OT and steroid hormones support the secretion of each other. On the other hand, hypersecretion of steroids can inhibit further surges of the steroidogenesis stimulator OT. Such positive and negative feedback regulation has been described previously for LH secretion [28, 29]. Our observations allow us to propose that similar relationships also hold between ovarian OT and steroid production.

In our experiments AVP inhibited bovine granulosa progesterone and stimulated estradiol secretion (Figs 3 and 4). The similar effects of AVP and AVT on progesterone secretion by human and porcine luteocytes were also described by other investigators, although stimulation of estradiol release was not observed in these conditions [8, 9, 24, 25]. All these data suggest that not only OT, but also AVP can control ovarian steroidogenesis. Although receptors to nonapeptide hormones have not been demonstrated in ovaries [3], the differences in AVP and OT effect patterns lead us to propose the existence of at least two types of such receptors—to OT and AVP. Both hormones can regulate ovarian steroidogenesis. But OT- and AVP-producing cells appear to have different responses to the feedback influence of steroid hormones. So, in contrast to OT, AVP release was not changed under the influence of steroids except for a slight AVP output activation by only one progesterone dose. No evidence for steroid influence on ovarian AVP is available in the present literature. The data reported here suggests the existence of not only a nonapeptide hormone influence on ovarian steroid release, but also of feedback interrelationships between steroids- and OT- (but not AVP) producing systems within the ovary.

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