# Multiple effects of neuropeptide Y, substance P and vasoactive intestinal polypeptide on progesterone and oxytocin release from bovine corpus luteum *in vitro*

**A. Miyamoto, A. Brückmann, H. von Lützow** and **D. Schams**

Institute of Physiology, Technical University of Munich, 8050 Freising-Weihenstephan, Germany

(A. Miyamoto is now at Department of Animal Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080, Japan)

(Requests for offprints should be sent to D. Schams)

RECEIVED 13 November 1992

## ABSTRACT

Recent observations indicate that the rat ovary receives not only adrenergic but also peptidergic innervation. In ruminants, there are few data available on the extent of a possible direct regulation of the peptidergic innervation of the ovary including the corpus luteum (CL). The direct effects of neuropeptide Y (NPY), substance P (SP) and vasoactive intestinal polypeptide (VIP) on the release of progesterone and oxytocin from mid-luteal phase CL (days 8–12) were examined *in vitro*. A possible direct neural influence might provide a sensitive short-term control. Long-term as well as short-term effects were assessed using both a serum-reduced luteal cell culture and a microdialysis system (MDS) of luteal tissue. In the long-term experiments, luteal cells were preincubated from the start of the culture for 48 h with NPY, SP and VIP (10 pmol/l–100 nmol/l). During the following 4 h the neuropeptides showed a dose-dependent stimulation of progesterone release, but there was no effect on oxytocin release. LH showed a synergistic effect with NPY, SP and VIP on progesterone release. In the short-term experiments, the neuropeptides were added 48 h after the start of the culture. All three peptides were most stimulatory to LH-supported progesterone release 30 min after addition, and the effect decreased greatly thereafter to the control level from 60 to 120 min. In contrast, LH alone induced the maximal progesterone stimulation at 120 min. In the MDS, a 30-min perfusion with NPY, SP or VIP (10 nmol/l, 100 nmol/l and 1 μmol/l) induced significant acute effects on progesterone and oxytocin release. At 10 and 100 nmol/l the neuropeptides slightly inhibited progesterone release, but stimulated oxytocin release. At 1 μmol/l, however, both progesterone and oxytocin release were stimulated.

We conclude that neuropeptides NPY, SP and VIP act directly on progesterone and oxytocin release from bovine CL *in vitro*. The results suggest that the hormonal function of bovine CL may be partly influenced by peptidergic as well as adrenergic innervation.

*Journal of Endocrinology* (1993) **138**, 451–458

## INTRODUCTION

The sympathetic innervation of the mammalian ovary has received increased attention during the last two decades (Capps *et al.* 1978; Moshin & Pennefather, 1979; Lawrence & Burden, 1980). Although the relative importance of ovarian innervation to endocrine or paracrine/autocrine control remains to be elucidated, the possibility that the bovine corpus luteum (CL) may be partly regulated by adrenergic innervation has been indicated (Condon & Black, 1976; Battista & Condon, 1986; Battista *et al.* 1987, 1989; Luck & Jungclas, 1987; Denning-Kendall *et al.* 1991; Kotwica *et al.* 1991). Using immunohistofluorescence in rats, it has been revealed that the ovary – in addition to adrenergic innervation – also receives peptidergic innervation (Ojeda & Urbanski, 1988). Neuropeptide Y (NPY) (Stjernquist *et al.* 1983; Papka *et al.* 1985; McDonald *et al.* 1987), substance P (SP) (Dees *et al.* 1985; Ojeda *et al.* 1985) and vasoactive intestinal polypeptide (VIP) (Dees *et al.* 1986; Klein & Burden 1988) have been located. These

  *Printed in Great Britain*
0022–0795/93/0138–0451 $08.00/0

immunoreactive fibres were observed in tissue surrounding blood vessels and in interstitial tissue, and associated with the thecal layers of developing follicles (Ahmed *et al.* 1986; Dees *et al.* 1986; McDonald *et al.* 1987). Moreover, VIP was shown to stimulate ovarian steroidogenesis (Davoren & Hsueh, 1985; Ahmed *et al.* 1986; Trzeciak *et al.* 1987), while NPY (Ojeda & Urbanski, 1988) and SP (Ojeda *et al.* 1985) had no effect. Therefore, the main function of NPY and SP in the ovary may be to regulate blood flow (Ojeda *et al.* 1988), although VIP may have a function in ovarian hormone secretion in rats. In ruminants, there are few data available relating to the possibility that the ovary may be directly regulated by peptidergic innervation.

We have therefore examined the direct effects of the neuropeptides NPY, SP and VIP on the release of progesterone and oxytocin from bovine CL *in vitro*. Because the infusion of NPY into the stalk-median eminence *in vivo* induces rapid release of luteinizing hormone-releasing hormone (LHRH), as shown in monkeys (Woller & Terasawa, 1991), a possible direct neural influence might provide a fine 'minute-to-minute' control (Ojeda & Urbanski, 1988). Thus short-term as well as long-term effects were assessed using both a luteal cell culture and a microdialysis system (MDS) of luteal tissue. The MDS has been assumed to be a valid addition to cell culture techniques in cases where cell-to-cell contact may be of importance (Miyamoto & Schams, 1991; Miyamoto *et al.* 1992; Sauerwein *et al.* 1992). Luteinizing hormone (LH) was also involved in the study with neuropeptides as it is an essential luteotrophic hormone (Hoffmann *et al.* 1974). We describe here the multiple effects of neuropeptides in the two in-vitro systems.

## MATERIALS AND METHODS

### Collection of CL

CL from German Fleckvieh (Simmental) cows were collected at a local slaughterhouse within 10–20 min of slaughter. The stage of the oestrous cycle was defined by macroscopic observation of the ovaries and the uterus (Miyamoto & Schams, 1991). Mid-luteal phase CL (days 8–12) were collected and washed several times with saline and processed for cell culture immediately. For in-vitro microdialysis, they were transported to the laboratory in Medium 199 (Sigma, Deisenhofen, F.R.G.) at 38 °C containing 10 mmol $NaHCO_3$/l, Earle's salts, 365 mg L-glutamine/l, 25 mmol Hepes/l, 5 g bovine serum albumin (BSA)/l, 60 mg penicillin/l, 100 mg streptomycin/l, 2 mg amphotericin/l and 56 mg ascorbic acid/l at pH 7·5 (M199).

### Tissue dissociation and cell preparation

A two-step perfusion procedure was used for the dissociation of the CL as described earlier (Miyamoto *et al.* 1992; Okuda *et al.* 1992). Briefly, Teflon microcapillaries (internal diameter 0·3 mm) (Bohlender, Lauda, Germany) were inserted into the ovarian arteries (*A. ovarica*) immediately after slaughter and the ovaries were perfused with an ice-cold EGTA buffer (0·1 mmol EGTA/l, 10 mmol Hepes/l, 140 mmol NaCl/l, 7·1 mmol KCl/l, pH 7·4) to remove blood and loosen endothelial cells. The ovaries were transported to the laboratory submerged in ice-cold EGTA buffer. The ovaries were then perfused first with EGTA buffer for 15 min, followed by a wash buffer (10 mmol Hepes/l, 140 mmol NaCl/l, 7·1 mmol KCl/l, 5·0 mmol $CaCl_2$/l) for 15 min. Finally, dissociation was obtained by perfusion with the wash buffer containing 0·05% collagenase (type I from *Clostridium hystolyticum*) and 0·1% BSA fraction V radioimmunoassay grade for up to 30 min. EGTA, Hepes and collagenase were purchased from Sigma, and NaCl, KCl and $CaCl_2$ were from Merck, Darmstadt, Germany. All perfusion buffers were maintained at 38 °C and were gassed with 5% $CO_2$ in 95% $O_2$. The CL were transferred to a Petri dish containing M199 (with Earle's salts and 25 mmol Hepes/l). Luteal cells were dispersed with combs from the CL matrix. Luteal cells from three CL were pooled and stirred for 10 min in M199 containing 0·05% collagenase and 0·005% DNase I (deoxyribonuclease I from bovine pancreas, type IV) (Sigma). After filtration through 250 µm, 150 µm and 105 µm polyester gauze, the cells were washed three times by centrifugation for 5 min at 50 *g* with culture medium (1 : 1 with Dulbecco's Modified Eagle's Medium and Ham's F-12 medium, supplemented with 0·1% BSA, 0·5 mg insulin/l, 60 mg penicillin/l, 100 mg streptomycin/l, 2 mg amphotericin-B/l, 2 mmol L-glutamine/l, 5 mg transferrin/l, and 5 µg sodium selenite/l). The luteal cells were counted in a haemocytometer and assessed for viability by exclusion of trypan blue (>85%). Contamination with endothelial cells and erythrocytes was <20%.

### Cell culture

The luteal cells ($2·5 \times 10^4$/0·5 ml) were incubated in 48-well culture plates (Costar, Cambridge, MA, U.S.A.) for up to 52 h in a humidified incubator in an atmosphere of 95% air and 5% $CO_2$ at 38 °C (Miyamoto *et al.* 1992). The culture plates were coated with culture medium supplemented with 10% calf serum (1–2 h) and then washed with twice the volume of the serum-free culture medium. The medium was changed every 24 h. For long-term effects, the medium was always supplemented with human NPY (SP Peninsula Laboratories, London, U.K.) or porcine VIP (Sigma) with or without 10 ng bovine LH/ml (NIH-B8; potency 1·03 NIH-LH-S1 U/mg). The neuropeptides were dissolved in acetic acid (10 mmol/l) at concentrations of 1 mmol/l. They were diluted with culture medium to a final concentration of at least 100 nmol/l. For control culture, acetic acid (1 μmol/l) was always added to the medium. The culture medium from the last 4 h after the second change of medium (between 48 and 52 h after the start of culture) was collected. For short-term effects, the neuropeptides and/or LH were added after 48 h of culture, and the medium was collected 30, 60 and 120 min thereafter. The collected medium was stored at −20 °C until assayed for progesterone and oxytocin.

### Microdialysis system *in vitro*

The MDS for bovine CL *in vitro* has been described in detail previously (Miyamoto & Schams, 1991). In brief, each CL was trimmed to a 10–12 mm cube, divided into four pieces through the face of the apex for three different doses and control groups, and penetrated by an 8–10 mm long dialysis capillary (Fresenius SPS 900 Hollow Fibres, Fresenius AG, St Wendel, Germany; cut off $M_r$ 1000 kDa). The capillary was glued at each end to a piece of silicone elastomer tubing (internal diameter 0·3 mm) which was attached and fixed onto the surface of the pieces of CL. For perfusion, one end of the tube was connected to a peristaltic pump, and the other was joined to the fraction collector. The prepared luteal pieces were then maintained in organ culture chambers (modified 2070 Tube; Falcon, Lincoln Park, NJ, U.S.A.) filled with 50 ml M199 in a water bath at 38 °C. M199 was continuously renewed at a flow rate of 15 ml/h. During the incubation, the luteal pieces were perfused with Ringer's solution at a flow rate of 1 ml/10 min for up to 8 h. These conditions were required for obtaining a constant release of progesterone and an LH stimulation response (Miyamoto & Schams, 1991). After 3 h of preincubation, a fraction of the perfusate was collected every 10 min for up to 5 h. After 1 h of basal release, NPY, SP or VIP were applied. To exclude the possibility that the pH of the infusate may affect hormone release, acetic acid (10 μmol/l) in Ringer's solution (equivalent to the highest dose of neuropeptides) was always run as a control; in no case was an effect observed. Effluent fractions after perfusion were stored at −20 °C until assayed for progesterone and oxytocin. The diffusion capacity of the membrane was about 1–3% of the infused concentration in the case of progesterone and oxytocin, and about 0·1% for larger proteins like LH.

### Determination of progesterone and oxytocin

Concentrations of progesterone and oxytocin were determined directly in the fractions from the MDS or in the media from cell culture with an enzyme immunoassay (Sauerwein *et al.* 1992) and a radioimmunoassay (Schams, 1983) respectively. The progesterone standard curve ranged from 1·27 to 318 nmol/l and the effective dose for 50% inhibition ($ED_{50}$) of the assay was 31·8 nmol/l. The oxytocin standard curve ranged from 0·625 to 80 pmol/l and the $ED_{50}$ of the assay was 14·2 pmol/l. The intra- and interassay variations were 4·0 and 8·8% for progesterone and 5·8 and 9·6% for oxytocin.

### Statistical analysis

The data from the cell cultures are shown as the mean ± S.E.M. of values obtained in three separate experiments in triplicate. The statistical significance of differences between control and treated groups was assessed by Student's *t*-test.

For the analysis of the MDS experiments, the concentrations of progesterone and oxytocin in the first six fractions (1 h perfusion with Ringer's solution only) from each experimental group were pooled to obtain baseline values (basal release of different CL: progesterone, 5·4–54·1 nmol/l; oxytocin, 1·2–46·9 pmol/l). All hormone concentrations in the fractions collected from each piece of CL were expressed as a percentage of the individual baseline (100%). The absolute progesterone and oxytocin concentrations (mean ± S.E.M.) are given in Fig. 3 legend. The maximal stimulation or inhibition of progesterone and oxytocin release by neuropeptides at different doses was tested at individual time-points throughout perfusion compared with the pooled baseline. Means were analysed by analysis of variance followed by Duncan's multiple range test.

## RESULTS

### Dose-dependent long-term effects of neuropeptides on progesterone release from luteal cells in culture

To investigate the long-term effects, neuropeptides (10 pmol/l–100 nmol/l) and/or LH (10 ng/ml) were added at the start of the culture (Fig. 1). NPY, SP (>100 pmol/l) and VIP (>1 nmol/l) showed a dose-dependent stimulation on progesterone release from luteal cells obtained at the mid-luteal stage between 48 and 52 h after the start of cell culture. There were significant synergistic effects of NPY and VIP with LH on progesterone release. SP showed an additive stimulation in the presence of LH. In contrast, LH alone or with neuropeptides had no effect on oxytocin

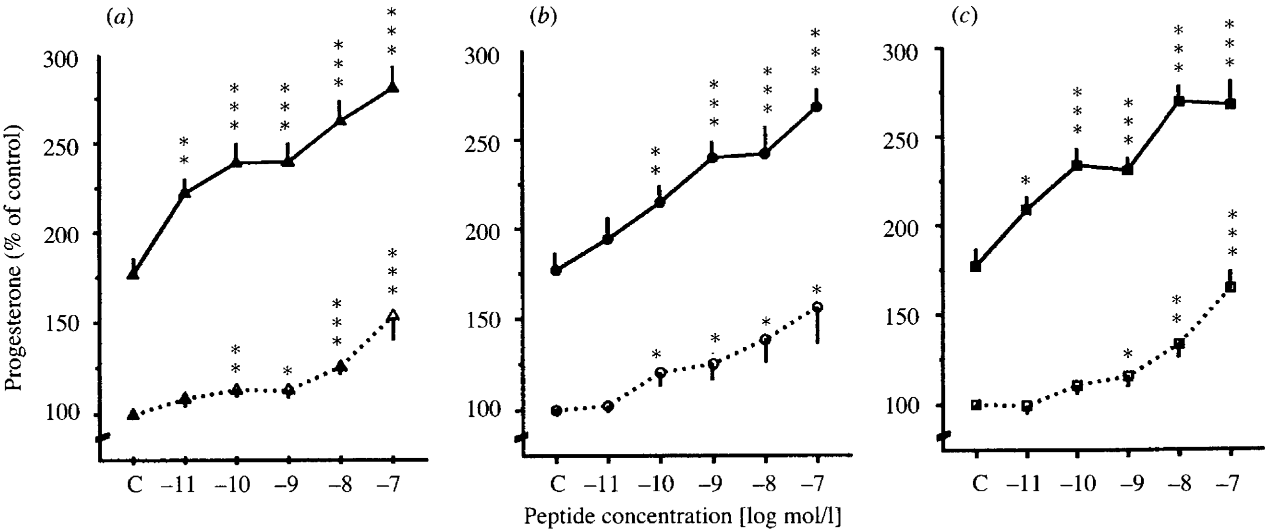


FIGURE 1. Dose-dependent long-term affects of (*a*) neuropeptide Y (triangles), (*b*) substance P (circles) and (*c*) vasoactive intestinal polypeptide (squares) on progesterone release from cultured bovine luteal cells. The cells were cultured with each neuropeptide in the absence (open symbols) or the presence of LH (10 ng/ml) (solid symbols) for 52 h. The culture medium was changed every 24 h. The medium from the last 4 h after the second change of medium (between 48 and 52 h after the start of the culture) was collected for hormone determination. Values are the mean ± S.E.M. of three separate experiments in triplicate (three corpora lutea for each experiment were pooled). Absolute progesterone concentrations in controls were 117 ± 11 nmol/l for basal release and 198 ± 11 nmol/l for LH-stimulated release respectively. *$P<0·05$, **$P<0·01$, ***$P<0·001$ compared with respective control (C) (Student's *t*-test).

secretion in the cell culture in the long term (data not shown).

**Short-term effects of neuropeptides on progesterone and oxytocin release from luteal cells in culture**

To investigate the short-term effects, neuropeptides (1 nmol/l) and/or LH (10 ng/ml) were added after 48 h of culture, and the medium was collected 30, 60 and 120 min thereafter (Fig. 2). Only VIP stimulated basal progesterone release during the whole period; NPY and SP were almost ineffective (Fig. 2*a*). Contrary to the basal progesterone release, the three neuropeptides were most stimulatory to LH-supported progesterone release at 30 min after addition and the effect decreased thereafter to control levels (Fig. 2*b*). The neuropeptides did not show any short-term effect on oxytocin release (Fig. 2*c* and *d*).

LH induced a different time-course of response to that of the neuropeptides; maximal progesterone stimulation by LH was observed 120 min after addition (Fig. 2*b*). LH had no effect on the release of oxytocin (Fig. 2*d*).

**Short-term effects of various doses of neuropeptides on progesterone and oxytocin release from microdialysed mid-luteal CL**

A 30-min perfusion with NPY, SP or VIP (10 nmol/l, 100 nmol/l and 1 µmol/l) induced significant acute effects on progesterone and oxytocin release from microdialysed CL (Fig. 3).

At doses of 10 and 100 nmol/l, the neuropeptides slightly inhibited progesterone release ($P<0·05$), but stimulated oxytocin release ($P<0·05$ for SP and VIP). There was considerable variation of oxytocin response between pieces of CL with these doses (not shown). At a higher dose of 1 µmol/l, the neuropeptides acutely stimulated both progesterone and oxytocin release ($P<0·05$).

## DISCUSSION

The present results provide the first evidence that the neuropeptides NPY, SP and VIP act directly – both acutely and chronically – on the release of progesterone and oxytocin from bovine CL *in vitro*. In serum-reduced cell culture, the neuropeptides were stimulatory to progesterone release, but not to oxytocin release. The long-term effects of VIP on progesterone release were comparable with those of rat granulosa cells (Ahmed *et al.* 1986) and ovaries (Davoren & Hsueh, 1985). Contrary to these and our own data, NPY and SP have been shown to inhibit progesterone and androstenedione release in porcine granulosa and luteal cells (Pitzel *et al.* 1991), in which a 48 h preincubation with 15% porcine serum-supplemented culture medium was used. Thus the

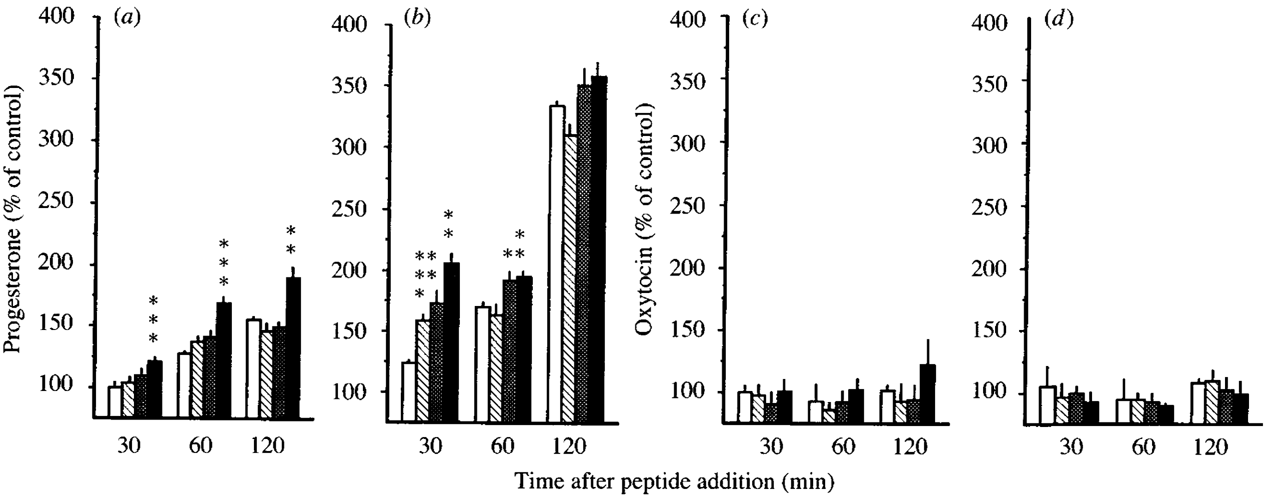


FIGURE 2. Short-term effects of neuropeptide Y (hatched bars), substance P (shaded bars) and vasoactive intestinal polypeptide (solid bars) on progesterone and oxytocin release from luteal cells in culture. (*a* and *c*) Neuropeptides (1 nmol/l) alone or (*b* and *d*) in combination with LH (10 ng/ml) were added after 48 h of culture, and the medium was collected after 30, 60 and 120 min. Absolute progesterone and oxytocin concentrations of the 30-min controls (baseline) were 73·4 ± 9 nmol/l and 52·2 ± 8·1 pmol/l. Values are the mean ± S.E.M. of three separate experiments in triplicates (three corpora lutea for each experiment were pooled). **P*<0·05, ***P*<0·01, ****P*<0·001 compared with respective controls (open bars) at the same time-point (Student's *t*-test).

opposite effects of NPY and SP may be attributed to different culture systems, i.e. a 2-day preincubation with serum or serum-coated cell culture plates. Alternatively, the opposite effects may reflect the different role of the peptides in different species, since NPY (Ojeda & Urbanski, 1988) and SP (Ojeda *et al.* 1985) using serum-free culture systems had no effects on ovarian steroidogenesis in rats. The fact that NPY, SP and VIP are more effective together with LH on progesterone release than NPY or VIP alone, suggests that these neuropeptides may act synergistically with LH on progesterone release.

In the short-term experiments, the most effective progesterone release to neuropeptides occurred within 30 min after addition of the peptide. It was apparent that the progesterone response to NPY, SP and VIP in the presence of LH was much higher than the response in the absence of LH. At this time, LH alone stimulated progesterone release by a mere 120%. Maximum progesterone release of 300% occurred 120 min after the addition of LH. Neuropeptides may amplify and synchronize the effect of LH.

The responses of CL to neuropeptides in the MDS revealed another profile. Neuropeptides at doses of 10 and 100 nmol/l partly suppressed progesterone release during peptide infusion, while oxytocin release was already acutely stimulated. The reason for the lower sensitivity of progesterone release is unclear. The higher dose of 1 µmol/l had acute stimulatory effects on both progesterone and oxytocin release. The CL response of oxytocin release to the neuropeptides was more variable at low doses. In monkeys, a similar tendency was observed for the release of LHRH after infusion of NPY into the hypothalamus (Woller & Terasawa, 1991). The doses in the present study (10 nmol/l–1 µmol/l) are consistent with those doses of NPY used for studies of LHRH release from the hypothalamus of rats *in vitro* (Crowley & Kalra, 1987; Sabatino *et al.* 1989) and in monkeys *in vivo* (Woller & Terasawa, 1991).

The major difference in the response of CL to neuropeptides between the two different systems appears to be a stimulation of oxytocin release observed only in the MDS with intact tissue but not in cell culture. Since oxytocin is mainly localized in and secreted by the large luteal cells (Rodgers *et al.* 1983*b*; Kruip *et al.* 1985; Fehr *et al.* 1987), one might speculate that the small luteal cells predominantly possess receptors for the neuropeptides and, therefore, isolated large luteal cells cannot respond to neuropeptides with oxytocin release. In general, small luteal cells gave a better progesterone release to hormones than large luteal cells, as shown for LH (Fitz *et al.* 1982; Rodgers *et al.* 1983*a*; Alila *et al.* 1988). In comparison with cell culture, intact cell-to-cell contact still exists in the MDS. Thus, small luteal cells may communicate the neuropeptide signal to large luteal cells by some intercellular transmitter stimulating the release of oxytocin.

In the cow, direct ovarian actions of catecholamines have been demonstrated *in vitro* using luteal tissues (Condon & Black, 1976; Godkin *et al.* 1977;

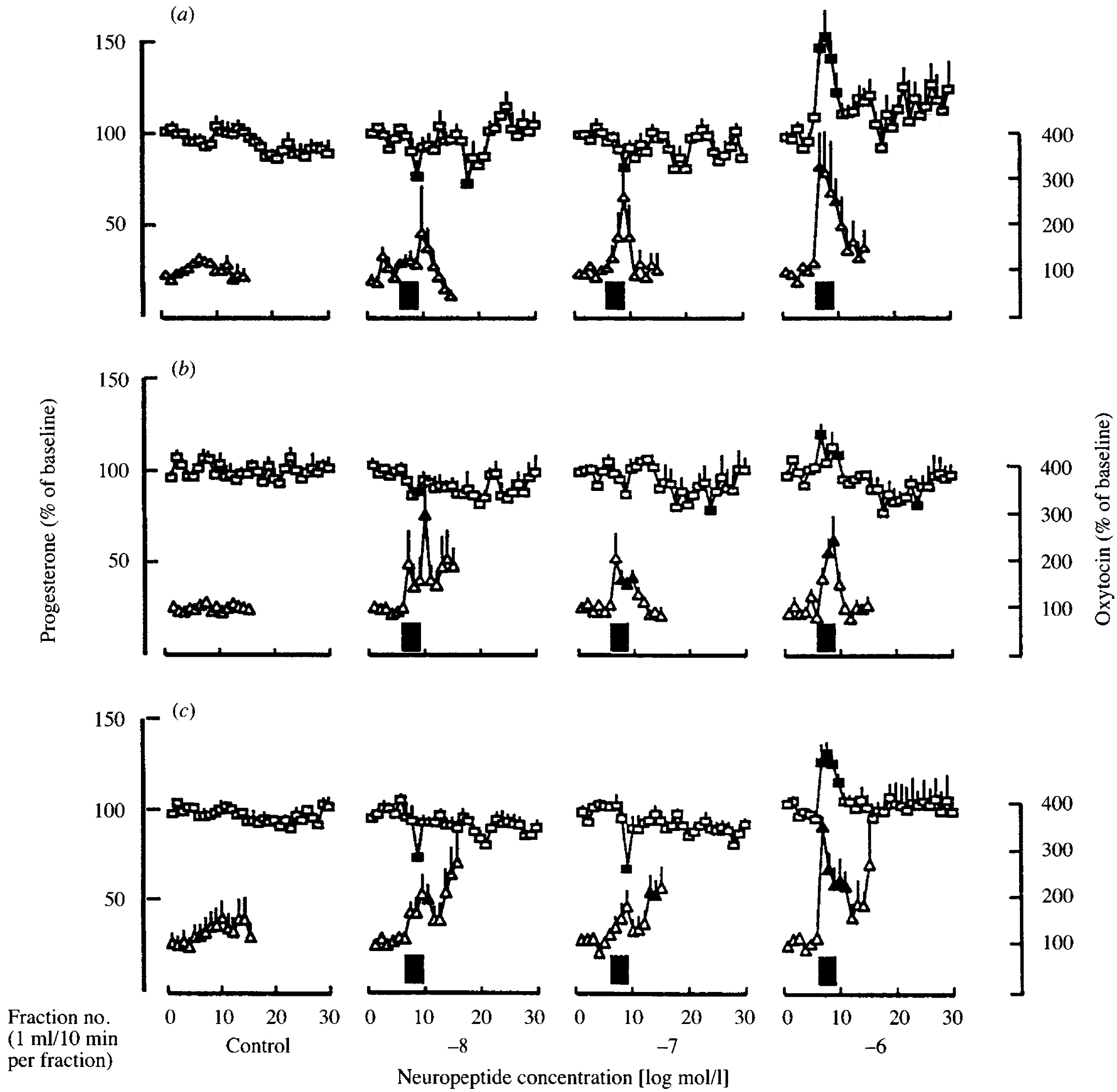


FIGURE 3. Short-term effects of (*a*) neuropeptide Y, (*b*) substance P and (*c*) vasoactive intestinal polypeptide on progesterone (rectangles) and oxytocin (triangles) release from microdialysed luteal tissue of the mid-luteal stage. All values are shown as a percentage of the respective baselines (means ± S.E.M.; $n$=6 corpora lutea/group) which were calculated from the first six fractions (perfused with Ringer's solution only). The solid symbols indicate a significant change ($P$<0·05) in progesterone and oxytocin secretion. The baseline absolute concentrations of progesterone and oxytocin were 23·5 ± 1·3 nmol/l and 11 ± 1 pmol/l respectively ($n$=72, mean ± S.E.M.).

Jordan *et al.* 1978; Battista & Condon, 1986) and granulosa cells (Luck & Jungclas, 1987, 1988). Bovine granulosa cells are stimulated by cholinergic neurotransmitters (Luck, 1990), as human granulosa cells are stimulated by the neurotransmitter serotonin (Bodis *et al.* 1992). The results found in the literature and from our study of NPY, SP and VIP suggest that the different ovarian compartments are under the direct control of neurotransmitters which are supplied by the ovary, or are influenced by locally circulating neurotransmitters. Neurotransmitters may act together, potentiate and/or prolong the effects of other neurotransmitters or hormones (like LH as shown in our cell culture experiments). Thus, external factors, such as stress or environmental change, may influence the ovary.

Luck (1990) proposed medium and long-acting persistent neurotransmitter receptor types in bovine granulosa cells and may be in luteal cells of the early luteal phase. Our data on both long- and short-term effects on hormone release may represent, in part, the multiple functions of neuropeptides in the ovary; they could act in a 'minute-to-minute' fashion and, at the same time, they would stimulate steroidogenesis in a 'day-to-day' fashion. The former may support a classical response of nerves, while the latter may support a possible intraovarian synthesis of neuropeptides as shown for VIP in rats (Gozes & Tsafriri, 1986) and for SP in swine (Pitzel *et al.* 1991).

In conclusion, the bovine CL responds to neuropeptides NPY, SP and VIP with the release of progesterone and oxytocin *in vitro*. The overall results suggest that the hormonal function of the bovine ovary may receive not only adrenergic innervation but also peptidergic innervation. To support this hypothesis, further biochemical and histochemical studies are needed as well as receptor-binding evidence in the bovine ovary and CL which must be collected in future investigations.

## ACKNOWLEDGEMENTS

We thank Mrs G. Schwentker and Mrs U. Eisenrieth for their technical assistance, Fresenius AG, St Wendel, Germany for the microcapillary membrane, and the National Hormone and Pituitary Program, University of Maryland School of Medicine and National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) for bovine LH (NIH-LH-B8). This research was partly supported by the Japan Society for the Promotion of Science.

## REFERENCES

Ahmed, C. E., Dees, W. L. & Ojeda, S. R. (1986). The immature rat ovary is innervated by vasoactive intestinal peptide (VIP)-containing fibers and responds to VIP with steroid secretion. *Endocrinology* **118,** 1682–1689.

Alila, H. W., Dowd, J. P., Corradino, R. A., Harris, W. V. & Hansel, W. (1988). Control of progesterone production in small and large bovine luteal cells separated by flow cytometry. *Journal of Reproduction and Fertility* **82,** 645–655.

Battista, P. J. & Condon, W. A. (1986). A role for alternative pathway catecholamines in the regulation of steroidogenesis in cow luteal cells. *Journal of Reproduction and Fertility* **78,** 275–280.

Battista, P. J., Poff, J. P., Deaver, D. R. & Condon, W. A. (1987). Biogenic amine regulation of bovine luteal progesterone production *in vivo*. *Journal of Reproduction and Fertility* **80,** 517–522.

Battista, P. J., Rexroad, C. E., Poff, J. P. & Condon, W. A. (1989). Support for a physiological role of endogenous catecholamines in the stimulation of bovine luteal progesterone production. *Biology of Reproduction* **41,** 807–812.

Bodis, J., Török, A., Tinneberg, H. R., Hanf, V., Hamori, M. & Cledon, P. (1992). Influence of serotonin on progesterone and estradiol secretion of cultured human granulosa cells. *Fertility and Sterility* **57,** 1008–1011.

Capps, M. L., Lawrence, I. E. & Buden, H. W. (1978). Ultrastructure of the cells of the ovarian interstitial gland in hypophysectomized rats. *Cell and Tissue Research* **193,** 433–442.

Condon, W. A. & Black, D. L. (1976). Catecholamine-induced stimulation of progesterone by the bovine corpus luteum *in vitro*. *Biology of Reproduction* **15,** 573–578.

Crowley, W. R. & Kalra, S. P. (1987). Neuropeptide Y stimulates the release of luteinizing hormone-releasing hormone from medial basal hypothalamus *in vitro*: modulation by ovarian hormones. *Neuroendocrinology* **46,** 97–103.

Davoren, J. B. & Hsueh, A. J. W. (1985). Vasoactive intestinal peptide: a novel stimulator of steroidogenesis by cultured rat granulosa cells. *Biology of Reproduction* **33,** 37–52.

Dees, W. L., Ahmed, C. E. & Ojeda, S. R. (1986). Substance P- and vasoactive intestinal peptide-containing fibers reach the ovary by independent routes. *Endocrinology* **119,** 638–641.

Dees, W. L., Kozlowski, G. P., Dey, R. & Ojeda, S. R. (1985). Evidence for the existence of substance P in the prepubertal rat ovary. II. Immunocytochemical localization. *Biology of Reproduction* **33,** 471–476.

Denning-Kendall, P. A., Wild, M. L. & Wathes, D. C. (1991). Regional differences in catecholamine concentrations in bovine ovaries analysed by high-performance liquid chromatography. *Journal of Endocrinology* **129,** 221–226.

Fehr, S., Ivell, R., Koll, R., Schams, D., Fields, M. & Richter, D. (1987). Expression of the oxytocin gene in the large cells of the bovine corpus luteum. *FEBS Letters* **210,** 45–50.

Fitz, T. A., Mayan, M. H., Sawyer, H. R. & Niswender, G. D. (1982). Characterization of two steroidogenic cell types in the ovine corpus luteum. *Biology of Reproduction* **27,** 703–711.

Godkin, J. D., Black, D. L. & Duby, R. T. (1977). Stimulation of cyclic-AMP and progesterone synthesis by LH, $PGE_2$ and isoproterenol in the bovine CL in vitro. *Biology of Reproduction* **17,** 514–521.

Gozes, I. & Tsafriri, A. (1986). Detection of vasoactive intestinal peptide-encoding messenger ribonucleic acid in the rat ovaries. *Endocrinology* **119,** 2606–2610.

Hoffmann, B., Schams, D., Bopp, R., Ender, M. L., Gimenez, T. & Karg, H. (1974). Luteotrophic factors in the cow: Evidence for LH rather than prolactin. *Journal of Reproduction and Fertility* **40,** 77–85.

Jordan, A. W., Caffrey, J. L. & Niswender, G. D. (1978). Catecholamine-induced stimulation of progesterone and adenosine 3′,5′-monophosphate production by dispersed ovine luteal cells. *Endocrinology* **103,** 385–392.

Klein, C. M. & Burden, H. W. (1988). Substance-P and vasoactive intestinal polypeptide (VIP)-immunoreactive nerve fibers in relation to ovarian postganglionic pericarya in para- and prevertebral ganglia: evidence from combined retrograde tracing and immunocytochemistry. *Cell and Tissue Research* **252,** 403–410.

Kotwica, J., Skarzynski, D. & Kotwica, G. (1991). Effect of norepinephrine on the release of progesterone and ovarian oxytocin in cattle. *Animal Reproduction Science* **26,** 179–191.

Kruip, T. A. M., Vullings, H. G. B., Schams, D., Jonis, J. & Klarenbeck, A. (1985). Immunocytochemical demonstration of oxytocin in bovine ovarian tissues. *Acta Endocrinologica* **109,** 537–542.

Lawrence, I. E. & Burden, H. W. (1980). The origin of the extrinsic adrenergic innervation to the rat ovary. *Anatomical Record* **196,** 51–59.

Luck, M. R. (1990). Cholinergic stimulation, through muscarinic receptors, of oxytocin and progesterone secretion from bovine granulosa cells undergoing spontaneous luteinization in serum-free culture. *Endocrinology* **126,** 1256–1263.

Luck, M. R. & Jungclas, B. (1987). Catecholamines and ascorbic acid as stimulators of bovine ovarian oxytocin secretion. *Journal of Endocrinology* **114,** 423–430.

Luck, M. R. & Jungclas, B. (1988). The time course of oxytocin secretion from cultured bovine luteal cells, stimulated by ascorbate catecholamines. *Journal of Endocrinology* **116,** 247–258.

McDonald, J. K., Dees, W. L., Ahmed, C. E., Noe, B. D. & Ojeda, S. R. (1987). Biochemical and immunocytochemical characterization of neuropeptide Y in the immature rat ovary. *Endocrinology* **120,** 1703–1710.

Miyamoto, A., Okuda, K., Schweigert, F. J. & Schams, D. (1992). Effects of basic fibroblast growth factor, transforming growth factor-β and nerve growth factor on the secretory function of the bovine corpus luteum *in vitro*. *Journal of Endocrinology* **135,** 103–114.

Miyamoto, A. & Schams, D. (1991). Oxytocin stimulates progesterone release from microdialyzed bovine corpus luteum *in vitro*. *Biology of Reproduction* **44,** 1163–1170.

Moshin, S. & Pennefather, J. N. (1979). The sympathetic innervation of the mammalian ovary. *Clinical and Experimental Pharmacological Physiology* **6,** 335–354.

Ojeda, S. R., Costa, M. E., Katz, K. H. & Hersh, L. B. (1985). Evidence for the existence of substance P in the prepubertal rat ovary. I. Biochemical and physiologic studies. *Biology of Reproduction* **33,** 286–295.

Ojeda, S. R. & Urbanski, H. F. (1988). Puberty in the rat. In *The Physiology of Reproduction*, 1699–1737. Eds E. Knobil & J. D. Neill. New York: Raven Press.

Okuda, K., Miyamoto, A., Sauerwein, H., Schweigert, F. J. & Schams, D. (1992). Evidence for oxytocin receptors in cultured bovine luteal cells. *Biology of Reproduction* **46,** 1001–1006.

Papka, R. E., Cotton, J. P. & Traurig, H. H. (1985). Comparative distribution of neuropeptide tyrosine-vasoactive intestinal polypeptide-substance P-immunoreactive, acetylcholinesterase-positive and noradrenergic nerves in the reproductive tract of the female rat. *Cell and Tissue Research* **242,** 475–490.

Pitzel, L., Jarry, H. & Wuttke, W. (1991). Direct effect of substance P and neuropeptide Y on the *in vitro* steroid release of porcine granulosa and luteal cells. *Endocrinology* **129,** 1059–1065.

Rodgers, R. J., O'Shea, J. D. & Findlay, J. K. (1983*a*). Progesterone production *in vitro* by small and large ovine luteal cells. *Journal of Reproduction and Fertility* **69,** 113–124.

Rodgers, R. J., O'Shea, J. D., Findlay, J. K., Flint, A. P. F. & Sheldrick, E. L. (1983*b*). Large luteal cells the source of luteal oxytocin in the sheep. *Endocrinology* **113,** 2302–2304.

Sabatino, F. D., Collins, P. & McDonald, J. K. (1989). Neuropeptide-Y stimulation of luteinizing hormone-releasing hormone secretion from the median eminence *in vitro* by estrogen-dependent and extracellular $Ca^{2+}$-independent mechanisms. *Endocrinology* **124,** 2089–2098.

Sauerwein, H., Miyamoto, A., Günther, J., Meyer, H. H. D. & Schams, D. (1992). Binding and action of insulin-like growth factors and insulin in bovine luteal tissue during the oestrous cycle. *Journal of Reproduction and Fertility* **96,** 103–115.

Schams, D. (1983). Oxytocin determination by radioimmunoassay III. Improvement to subpicogram sensitivity and application to blood levels in cyclic cattle. *Acta Endocrinologica* **103,** 180–183.

Stjernquist, M., Emson, P., Owman, C., Sjöberg, N. O., Sundler, F. & Tatemoto, K. (1983). Neuropeptide in the female reproductive tract of the rat. Distribution of nerve fibers and motor effects. *Neuroscience Letters* **39,** 279–284.

Trzeciak, W. H., Waterman, M. R., Simpson, E. R. & Ojeda, S. R. (1987). Vasoactive intestinal peptide regulates cholesterol side-chain cleavage cytochrome P-450 (P-450scc) gene expression in granulosa cells from immature rat ovaries. *Molecular Endocrinology* **1,** 500–504.

Woller, M. J. & Terasawa, E. (1991). Infusion of neuropeptide Y into the salk-median eminence stimulates *in vivo* release of luteinizing hormone-releasing hormone in gonadectomized rhesus monkeys. *Endocrinology* **128,** 1144–1150.