Vasoactive intestinal polypeptide in the PMSG-primed immature rat ovary and its effect on ovulation in the isolated rat ovary perfused *in vitro*

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Summary. The immature rat ovary contains VIP immunoreactive nerve fibres sparsely distributed around blood vessels, in the interstitial gland and around follicles. The VIP concentration, measured radioimmunologically, decreased significantly after PMSG treatment (10 i.u.), probably due to ovarian enlargement and oedema, while the total VIP content (total of 0.12 pmol in both ovaries) did not change after PMSG priming. The ovulatory effect of VIP was studied using in-vitro perfused ovaries from immature 28-day-old rats primed with 10 i.u. PMSG. In all ovaries perfused, VIP (10^{-7} M) induced ovulations with a rate of 2.33 ± 0.56 . The ovulation rate was significantly lower than that of ovaries stimulated by LH (0.1 µg/ml) (5.20 ± 0.86 ovulations per ovary). No synergistic effect on the ovulation rate was seen when LH and VIP were administered together (5.20 ± 0.49 ovulations per ovary). The results suggest that the neuropeptide VIP may represent one of the local factors involved in the ovulation process.

Keywords: VIP; rat ovary; ovulation

Introduction

Vasoactive intestinal polypeptide (VIP) is a 28-amino acid peptide located in central and peripheral nerves where it appears to function as a neurotransmitter (Hökfelt *et al.*, 1984; Hansen *et al.*, 1986). VIP-containing nerves innervate many different tissues, thus having a wide variety of actions, including vasodilatation, smooth muscle relaxation in the respiratory, gastrointestinal and urogenital tracts, hormone release from pancreas and facilitatory effects on secretion from salivary glands. The VIP immunoreactive nerve fibres reach the ovary via the suspensory ligament (Dees *et al.*, 1986) and are located around blood vessels, in the interstitial gland and in the follicular wall of the ovary (Kannisto *et al.*, 1986; Ahmed *et al.*, 1986). The existence of VIP mRNA in the rat ovary also suggests a local synthesis of VIP in the gland (Gozes & Tsafirri, 1986).

The fact that VIP influences smooth muscle cells in the follicular wall (Schmidt *et al.*, 1988a), ovarian steroid production (Fredericks *et al.*, 1983; Davoren & Hsueh, 1985; Ahmed *et al.*, 1986; Ottesen *et al.*, 1986) and plasminogen activator activity in rat granulosa cells and cumulus-oocyte complexes (Liu *et al.*, 1987) indicates that VIP may be involved in the ovulatory process. VIP immunoreactive fibres have been demonstrated in the hypothalamus and the anterior pituitary and there is reason to believe that VIP is involved in the regulation of prolactin secretion (Kato *et al.*, 1978; Ottesen *et al.*, 1981). To study the direct effect of VIP at the ovarian level, thus avoiding

systemic effects of the peptide, a well established in-vitro perfusion system for the PMSG-primed immature rat ovary was used (Koos *et al.*, 1984; Schmidt *et al.*, 1986). Immunocytochemical and immunochemical investigation of the immature rat ovary was performed to elucidate any fluctuations in VIP fibre localization and VIP content after the PMSG treatment.

Materials and Methods

Animals. Immature female Sprague-Dawley rats (Anticimex, Stockholm, Sweden) were housed under controlled conditions of 14 h light (05:00–19:00 h) and 10 h darkness and fed a standard diet of pellets and water *ad libitum*. At 26 days of age the rats were primed in the morning by a s.c. injection of 10 i.u. PMSG. On the morning of Day 28, before the endogenous LH surge which occurs between 15:00 and 18:00 h of that day (Ekholm & Hillensjö, 1982), the animals were used in different experiments as described below.

Immunohistochemistry. Four rats were killed by cervical dislocation at 19:00–20:00 h on Day 28: 2 of the rats had been primed with 10 i.u. PMSG on Day 26, while the other two rats were without PMSG treatment. The ovaries were rapidly removed and placed in a mixture of 2% paraformaldehyde and 15% saturated aqueous picric acid solution in 0.1 M-phosphate buffer at pH 7.2, 20°C for 24 h. The preparations were repeatedly rinsed in Tyrode's solution containing 10% sucrose for 48 h. The preparations were then frozen in a cryostat (–20°C) and sections of 12 µm were produced. The sections were placed onto chromalum-coated glass microscope slides for immunostaining. All sections were first incubated in antiserum against VIP (raised in guinea-pig, dilution 1:1280, Milab, Malmö, Sweden, code GP 8701) over-night at +4°C according to the indirect immunofluorescence technique of Coons *et al.* (1955). For visualization of the primary reaction, the sections were then covered with goat anti-guinea-pig IgG (Sigma, St Louis, MO, USA) conjugated to fluorescein isothiocyanate (FITC), dilution 1:80, for 45 min at +20°C. The antibodies were diluted in 10 mM-phosphate buffered saline (PBS), pH 7.3, with 0.5% Triton X-100. The sections were rinsed in PBS between every incubation as well as at the end of the last incubation. The sections were viewed with a Zeiss photomicroscope equipped with a 490 nm excitation filter to visualize FITC-conjugated antibodies. Control specimens were exposed to antisera that had been preabsorbed with excess amount of antigen (10–100 µg pure VIP per ml diluted antiserum). The slides were examined in a fluorescence microscope fitted with appropriate filter setting for observation of FITC (excitation 490 nm, emission 520 nm). No staining was observed in these control specimens.

Chemical determination. PMSG-treated immature rats were killed by cervical dislocation at 08:00–10:00 h and 19:00–20:00 h on Day 28 and at 04:00–05:00 h on Day 29 (7–8 animals were killed at each time). One additional group, consisting of 8 rats which had not received any PMSG injections, were killed at 08:00–10:00 h on Day 28. Both ovaries from each animal were immediately removed, weighed together, frozen on solid CO₂ and stored at –80°C until extraction. The extraction procedure used in this study is a modification of the initial method published by Fahrenkrug *et al.* (1978) for extraction of VIP. The frozen tissue was cut into small pieces on solid CO₂ transferred to acid-ethanol at –20°C [final concentration 68% (v/v) ethanol with 0.1 M-HCl], and incubated overnight at 4°C. On the next day centrifugation at 10 000 g was performed for 20 min at 4°C. The supernatant was stored at 4°C. The pellet was washed with 1 ml acid-ethanol, and the sample was centrifuged for 10 min at 4°C. The first and second supernatants were pooled and neutralized with an aqueous ammonia solution and centrifuged at 3000 g for 10 min at 4°C. Protein in the new supernatant was precipitated by adding 2 volumes of absolute ethanol and 4 volumes of diethyl ether. The precipitate that formed during incubation overnight at 4°C was dried by evaporation. The specimens were redissolved in assay buffer and the VIP content was determined according to Fahrenkrug & Schaffalitzky de Muckadell (1977). The sensitivity of the assay was 3.3 pmol/l; intra- and interassay coefficients of variations were approximately 10 and 15%, respectively. Samples (1.5 ml) from the perfusion medium were withdrawn in some experiments for VIP analysis. The samples were stored at –18°C before radioimmunological determination of VIP as described in detail by Helm *et al.* (1987). The sensitivity of the assay was 3.8 pmol/l; intra- and interassay variations were 4% and 8.5% respectively.

In-vitro perfusion. The surgical procedure and preparation of the ovaries have been described in detail elsewhere (Koos *et al.*, 1984). The rats (28 animals) were anaesthetized with pentobarbitone sodium (40 mg/kg body wt i.p.; Mebumal: ACO, Solna, Sweden). After injection of 100 IU heparin (Heparin sodium: Kabi Vitrum, Stockholm, Sweden) into a tail vein the abdomen was opened. The abdominal part of the aorta and vena cava were cannulated with infusion catheters (Viggo, Helsingborg, Sweden) and were dissected free together with the right ovary and its ovarian artery and vein. The preparation was then removed and connected to the perfusion apparatus.

The recirculating perfusion system is thoroughly described and has been used in several experiments in our laboratory (Schmidt *et al.*, 1985, 1986, 1988b, c). Three ovaries were perfused simultaneously in three identical perfusion systems. The medium was M 199 with Earle's salts and L-glutamine (Gibco Bio-Cult, Paisley, UK). Bicarbonate (0.026 M) was added to obtain a pH of 7.4 when the medium was continuously oxygenated with a gas mixture of 5% CO₂ and 95% O₂. The medium also contained 75 µg benzylpenicillin G/ml, 50 µg streptomycin/ml, 0.2 IU heparin/ml, 0.02 IU insulin/ml and 2% fatty acid-free bovine serum albumin (BSA, Sigma). The perfusion medium was maintained at 37°C and the pressure was adjusted to 70–90 mmHg, giving a flow of the perfusate of about 0.8 ml/min.

After 1 h of perfusion, test substances were added to the perfusate. In experiments with LH, 0.1 µg/ml of the hormone was added to the medium.

VIP (immediate concentration in the system of 10^{-7} M or 10^{-9} M) was administered after 1 and 8 h of perfusion. To estimate the concentration of VIP in the perfusate, samples of medium (1.5 ml) were taken 1 h after the accommodation time and every second hour thereafter. Every sample was replaced with the same amount of fresh medium. The last sample was taken at 06:00 h on the following morning, approximately 16–18 h after the start of perfusion. The ovary was removed from the perfusion system and observed for follicular ruptures with the aid of a Zeiss OPM stereomicroscope at $\times 15$ –30 magnification. The medium from the organ chamber was collected and centrifuged (1600 g) and the pellet examined by phase-contrast microscopy for the presence of oocytes.

Drugs. Ovine luteinizing hormone (NIAKKD-oLH-25) was purchased from NIH, Bethesda, MD, USA. Pregnant mare serum gonadotrophin (PMSG) and vasoactive intestinal polypeptide (VIP) were obtained from Sigma.

Statistics. The number of ovulations per ovary and VIP determinations in ovarian tissue and perfusion medium were expressed as mean values $\pm$ s.e.m. The significance level in the statistical comparison between mean values of the perfused ovaries was calculated according to Student's *t* test for unpaired observations while mean values from ovarian VIP content were compared with analysis of variance (ANOVA) followed by the Mann-Whitney U-test.

Results

Presence of VIP on the rat ovary

The distribution of VIP immunoreactivity is illustrated in Figs 1 and 2. Ovaries from the untreated immature rats contained a sparse number of VIP fibres running in the proximity of blood vessels as well as in the ovarian stroma and around follicles. In ovaries from the PMSG-primed rats excised in the evening of Day 28 (19:00–20:00 h) hardly any VIP fibres were observed.

As shown in Table 1, ovaries from PMSG-primed rats of the same age as controls and killed at 08:00–10:00 h were more than twice as heavy. The ovarian weight of PMSG-treated rats increased still more in the evening (19:00–20:00 h) and in the following morning (04:00–05:00 h). The VIP concentration (Table 1) was significantly higher in the untreated group when compared with the PMSG-treated groups. The total VIP content in both ovaries (pmol) (Table 1) showed only minor, statistically non-significant, variations between groups.

Ovaries perfused *in vitro*

No significant changes in flow rate and perfusion pressure were observed after the addition of any of the substances to the perfusion medium. An oedematous swelling of the ovary was noticed with the stereomicroscope as well as with the naked eye within 3 h of the LH and VIP administration. No ovulation was seen during the first 9 h of perfusion. Some of the oocytes recovered in the morning were degenerated.

No ovulations occurred in the control group perfused with saline alone (Table 2, Group 1), whereas all ovaries stimulated by LH and LH + VIP had about the same number of ovulations (Table 2, Group 2 and 3). Administration of VIP after 1 h and 8 h of perfusion in a dose corresponding to an immediate concentration in the medium of 10^{-7} M led to a gradual decrease of the peptide in the perfusion medium (Fig. 3). VIP alone (immediate concentration 10^{-7} M) induced ovulation in all ovaries perfused (Table 2, Group 4) while a very much lower concentration of VIP caused ovulation in 3 out of 6 ovaries (Table 2, Group 5). The ovulation frequency of the two VIP doses tested was, however, significantly lower than that of LH-stimulated ovaries (Table 2, Group 2).

Discussion

The present study revealed the presence of VIP nerves in close connection with blood vessels, in the interstitial tissue and around ovarian follicles in the untreated rats. The existence and localization

Fig. 1. VIP immunofluorescence in nerve fibres running in the ovarian interstitial tissue and around blood vessels. PMMSG-treated, 28-day-old rat. $\times 250$.

Fig. 2. VIP immunoreactive varicose nerve fibre running in the interstitial tissue and into the theca externa of the follicle. Untreated, 28-day-old rat. $\times 250$.

Table 1. Ovarian weight, VIP concentration and total VIP content (both ovaries from each animal pooled) in PMSG-treated animals before (08:00–10:00 h) and after the endogenous LH surge (19:00–20:00 h), and after ovulation (04:00–05:00 h)

	Control (08:00–10:00 h)	PMSG (08:00–10:00 h)	PMSG (19:00–20:00 h)	PMSG (04:00–05:00 h)
No.	8	7	8	7
Ovarian weight (mg wet wt)	17.6 ± 0.8	40.3 ± 1.2*	61.1 ± 4.5*	74.8 ± 5.2*
Ovarian VIP conc. (pmol/g)	7.4 ± 0.4**	3.3 ± 0.1	2.1 ± 0.1	1.7 ± 0.1
Total VIP content (pmol)	0.128 ± 0.002	0.134 ± 0.004	0.126 ± 0.002	0.128 ± 0.003

Values are mean ± s.e.m. for 7–8 animals.

* $P < 0.01$, ** $P < 0.001$ compared with untreated animals (control).

Fig. 3. Concentration of VIP in the medium during in-vitro perfusion of the rat ovary following administration of VIP (10^{-7} M) 1 h and 8 h after the start of perfusion. Values are mean ± s.e.m., $n = 5$.**Table 2.** Ovulation rates in rat ovaries perfused *in vitro* in the presence of VIP and/or LH (0.1 µg/ml)

Group	Treatment	Ovaries ovulating/ no. perfused	Ovulations per ovary
1	Control	0/6	0
2	LH	5/5	5.20 ± 0.86
3	LH + VIP (10^{-7} M)	5/5	5.20 ± 0.49
4	VIP (10^{-7} M)	6/6	2.33 ± 0.56*
5	VIP (10^{-9} M)	3/6	0.50 ± 0.22**

Values are mean ± s.e.m., $n = 6$ for Groups 1, 4 & 5 and $n = 5$ for Groups 2 & 3.

* $P < 0.02$ compared with Group 2.

** $P < 0.001$ compared with Group 2.

of VIP fibres in the ovary have been reported by others (Larsson *et al.*, 1977; Håkanson *et al.*, 1982; Kannisto *et al.*, 1986; Ahmed *et al.*, 1986) and indicates that VIP may have an influence on ovarian function. There was a sparse number of VIP fibres in the ovary compared to other organs in the female genital tract (Helm *et al.*, 1981; Ahmed *et al.*, 1986; Kannisto *et al.*, 1986; Owman & Stjernquist, 1988), and ovarian enlargement after PMSG treatment is probably the reason why no VIP nerves could be discovered in ovaries from the PMSG-primed rats. A depletion of VIP from nerve fibres and ovarian tissue is unlikely since the total ovarian VIP content did not decrease after PMSG treatment. PMSG is able to induce a synchronized development of a first generation of preovulatory follicles with a subsequent endogenous gonadotrophin surge leading to ovulation (Ekholm *et al.*, 1981, 1982). The extraction method used in this study has been evaluated to be at least as effective as the more commonly used 'boiled water-acetic acid' method (Fahrenkrug *et al.*, 1978). Accordingly, the ovarian VIP concentration was of the same magnitude or somewhat higher than reported earlier (Ottesen *et al.*, 1981; Huang *et al.*, 1984; Ahmed *et al.*, 1986) and did not change significantly before and after the LH surge or after ovulation.

Ovaries were perfused with two different concentrations of the neuropeptide: 10^{-9} M-VIP corresponds well with basal plasma levels (for references, see Ottesen, 1983), while 10^{-7} M-VIP is known to cause certain biological effects *in vitro* (Ottesen, 1983; Ojeda *et al.*, 1989). All glassware was siliconized and the tubing was made of Teflon, Viton and silicone to reduce adsorption of steroids and other substances during perfusion (for further details see Janson *et al.*, 1982). However, VIP determinations on the perfusion medium showed a decrease in VIP concentration during perfusion, probably due to a certain degree of adsorption and metabolism of the peptide (Ahmed *et al.*, 1986). The VIP dose was therefore repeated after 8 h of perfusion.

In the present study VIP alone induced ovulation in the in-vitro perfusion system. The ovulatory effect was dose-dependent, the higher dose (10^{-7} M) causing approximately 45% of the ruptures induced by LH. The ovulations were probably due to the presence of the peptide since no follicle ruptures occurred in the perfused control ovaries (see also Koos *et al.*, 1984; Schmidt *et al.*, 1986, 1988b, c). No synergistic effects on the ovulation rate were seen when LH was present together with VIP in the perfusion medium. This is in accordance with results from in-vivo experiments with rabbits by Fredericks *et al.* (1983). Intravenous infusion of VIP in the rabbit during oestrus or after ovulation induction with human chorionic gonadotrophin (hCG) increased progesterone secretion, but no effect was seen upon fertility or the number of corpora lutea formed.

There are several ways in which VIP may have a direct ovarian influence on the ovulatory process. Within 3 h of perfusion, ovaries treated with LH or VIP showed an oedematous swelling while control ovaries maintained approximately their original size. The vascular changes occurring in the ovary and especially in the mature follicles after the LH surge include vasodilatation and increased permeability of the capillaries, leading to extravasation of serum proteins, increasing interstitial oedema and, hence, a greater availability of hormones and other circulating agents to the ovary. The appearance of fenestrations and intercellular gaps between adjacent endothelial cells (Okuda *et al.*, 1983) as ovulation approaches makes a free connection between the capillary lumen and the follicular antrum possible (Bjersing & Cajander, 1974). These changes and the invasion of leucocytes, basophils and platelets around mature follicles has been compared to an inflammatory reaction (Espey, 1980). Many vasoactive substances such as histamine, 5-hydroxytryptamine and prostaglandins can take part in this event (Espey, 1980; Schmidt *et al.*, 1988b, c). The vasodilatory action of VIP (Said, 1980) and the presence of VIP-containing nerves around arterioles in the ovary, as documented in this study and reported by others (Larsson *et al.*, 1977; Helm *et al.*, 1981; Ahmed *et al.*, 1986), will probably contribute to the preovulatory ovarian hyperaemia. VIP-containing nerves also innervate the theca externa of the follicular wall (Ahmed *et al.*, 1986; Kannisto *et al.*, 1986). The neuromuscular complex of the follicle wall (Burden, 1978; Owman *et al.*, 1979) is suggested as being involved in the ovulatory process (Owman *et al.*, 1979). VIP may influence the follicle wall indirectly by modifying the nerve-mediated effect on the smooth muscle cells in the theca externa (Misbahuddin *et al.*, 1986; Yau *et al.*, 1986) or by a direct relaxant action on the cells (Schmidt *et al.*, 1988a).

VIP is also known to stimulate ovarian steroid secretion in the rabbit (Fredericks *et al.*, 1983) and rat (Davoren & Hsueh, 1985; Ahmed *et al.*, 1986), with cAMP as one of the intracellular messengers (Davoren & Hsueh, 1985; Törnell *et al.*, 1988). Progesterone has been shown to induce ovulation in hypophysectomized rats (Takahashi *et al.*, 1974) as well as in explanted hamster ovaries *in vitro* (Baranczuk & Fainstat, 1976), and it facilitates ovulation in incubated rabbit follicles (Testart *et al.*, 1983) and in rabbit ovaries perfused *in vitro* (Holmes *et al.*, 1985). Forskolin, an activator of adenylate cyclase, is able to induce ovulation in the rabbit ovary perfused *in vitro* (Holmes *et al.*, 1986), and in the perfused rat ovary both forskolin and cAMP induce ovulation (Brännström *et al.*, 1987). VIP also stimulates plasminogen activator activity in granulosa cells and cumulus-oocyte complexes in the rat (Liu *et al.*, 1987). Since plasminogen activator activity, with generation of proteolytic enzymes, is involved in the ovulatory process (Akazawa *et al.*, 1983; Reich *et al.*, 1985), it is possible that this is yet another way whereby VIP contributes to the process of ovulation.

This work was supported by the Swedish Medical Research Council (No. 14x-732/5680) the Faculty of Medicine, University of Lund, and the Thelma Zoegas Foundation, Helsingborg (Sweden). We thank Ms Mary-Ann Sällström for skilful technical assistance.

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Received 9 February 1990