Involvement of local adrenergic receptors in the process of ovulation in gonadotrophin-primed immature rats

P. Kannisto, Ch. Owman and B. Walles

Department of Histology, University of Lund, S-223 62 Lund, Sweden

Summary. Immature female rats were primed with 4 i.u. PMSG at 08:00 h of Day 26. This results in ovulation in the morning of Day 29. The number of ovulations was counted in terms of newly formed corpora lutea in the morning of Day 30. Various adrenergic drugs were delivered into the ovarian bursa bilaterally in the afternoon of Day 27 to study their effect on ovulation. A methyl cellulose gel solution was used as vehicle to minimize leakage from the bursa. Noradrenaline, terbutaline and 4-aminopyridine significantly enhanced the number of corpora lutea compared to control ovaries injected with gel vehicle alone. The effect of terbutaline was counteracted by propranolol. Phentolamine partly blocked the noradrenaline-induced enhancement and the antagonist alone significantly reduced the number of ovulations. The results indicate that stimulation of α -adrenergic receptors (probably via actions in the follicle wall) as well as β -receptors (influencing steroid-producing cells) may interfere with the ovulation process.

Introduction

The mammalian ovary is supplied with an extensive system of non-vascular adrenergic nerves, which are present within the wall of follicles in various stages of development (for references, see Stefenson, Owman, Sjöberg, Sporrang & Walles, 1981). The sympathetic nerve endings innervate actomyosin-containing cells in the theca externa layer of the follicle wall (Amsterdam, Lindner & Gröschel-Stewart, 1977; Walles, Gröschel-Stewart, Owman, Sjöberg & Unsicker, 1978). Local electrical stimulation of these adrenergic fibres induces a contraction of the wall mediated by α -adrenergic receptors (Walles, Owman & Sjöberg, 1982).

There is reason to believe that the intra-ovarian sympathetic nerves, through their influence on follicular contractility, may affect the mechanism of ovulation (Burden, 1978; Mohsin & Pennefather, 1979; Owman, Sjöberg, Wallach, Walles & Wright, 1979). Blockade of the ovarian α -receptors by phenoxybenzamine leads to a markedly reduced incidence of ovulation in gonadotrophin-primed rats (Walles *et al.*, 1977a). The number of ruptured follicles has also been modified by interfering with local adrenergic mechanisms in rabbit ovaries perfused *in vitro* (Kobayashi *et al.*, 1983).

The aim of the present study was to analyse changes in the incidence of ovulation, reflected in the number of newly formed corpora lutea, after local administration of sympathomimetic and sympatholytic agents into the ovarian bursa of immature rats treated with pregnant mare serum gonadotrophin (PMSG). PMSG priming of rats gives well-characterized responses (Nuti, Sridharan & Meyer, 1975), providing a defined, single generation of corpora lutea, which can be directly counted in a stereomicroscope.

Materials and Methods

Animals. Studies were performed on 163 immature female Sprague–Dawley rats, aged 26 days at the beginning of the experiment and weighing 60–70 g. The animals were maintained at normal

room temperature (+20°C) with a light schedule of 12 h light/12 h dark, the light cycle starting at 06:00 h. They were fed standard pellet food and received tap water *ad libitum*.

Ovulation. To obtain a well-defined, single phase of follicular ruptures, the animals were primed with 4 i.u. PMSG s.c. at 08:00 h of Day 26 (Text-fig. 1). This results in a preovulatory surge of gonadotrophins in the afternoon of Day 28, followed by ovulation in the morning of Day 29 (Herlitz, Koch, Khan & Ahrén, 1976; Khan *et al.*, 1979; Ahrén, Khan & Selstam, 1981). The animals were anaesthetized with 5% chloral hydrate i.p. (0.7 ml/100 g body wt) at 10:00–12:00 h on Day 30, the ovaries were dissected out, and the animal was killed by bleeding. The number of ruptured follicles was counted, in terms of newly formed corpora lutea, in coded ovaries by two persons separately, using a Zeiss OPMII stereomicroscope at $\times 15$ –30 total magnification. The results were confirmed by examination of 10 μ m paraffin-wax sections of the ovaries stained with haematoxylin and eosin. The corpora lutea always appeared normal, with no signs of oocyte retention.

Text-fig. 1. Experimental protocol used for rats in the present study. The times between PMSG priming, drug injection and counting of corpora lutea are related to the endogenous gonadotrophin surge and ovulation.

Experimental. The various active compounds to be tested were dissolved in saline (9 g NaCl/l) which, for noradrenaline and isoprenaline, also contained 0.2 mg ascorbic acid/ml as antioxidant. They were administered locally into the ovarian bursa between 13:00 and 15:00 h on the day after PMSG priming (Text-fig. 1). To minimize leakage from the bursa and to allow for a protracted effect of the active compound the drug solution was carried in a gel: 1 ml of the solution was mixed in 9 ml of a 4.5% solution of methyl cellulose gel (Hypromellose 4000: ACO, Solna, Sweden). Under methohexital sodium anaesthesia (Brietal: Lilly, Indianapolis, U.S.A.; 0.4 ml i.p. of a 10 mg/ml solution), each animal received a slow injection of 20 μ l gel solution on each side into the ovarian bursa exposed from a dorsal route. A 0.4 mm o.d. injection needle was used, and care taken not to damage the ovary during the injection. Control animals studied simultaneously were given bilateral injections of gel solution in the same amount (20 μ l into each ovarian bursa) but without active compound.

Control experiments with noradrenaline and terbutaline were carried out to evaluate any systemic effects the locally injected compounds might have through direct diffusion into the circulation or via inadvertent leakage from the ovarian bursa. The previous time protocol after PMSG priming was followed (Text-fig. 1). Three groups with 5 animals in each received bilateral injections of 20 μ l gel vehicle alone into the ovarian bursa. Animals in one of the groups also received 40 μ l i.m. of the gel solution containing 10^{-5} M-noradrenaline, and the other was given 10^{-5} M-terbutaline. The number of ruptured follicles was counted as described above.

In an attempt to evaluate any traumatic effect by the injections of gel solutions into the ovarian bursa, 4 animals received 20 μ l gel carrier bilaterally into the bursa according to the usual schedule

(Text-fig. 1) and were compared with 4 PMSG-primed animals in which the ovaries were exposed but no injections were made into the bursa.

Drugs. The following active compounds were used: PMSG (1500–3000 i.u./mg), L-arterenol hydrochloride, L-isoprotorenol hydrochloride and 4-aminopyridine (all from Sigma, St Louis, MO, U.S.A.), terbutaline sulphate (Bricanyl: Draco, Lund, Sweden), phentolamine (Regitin: Ciba-Geigy, Basel, Switzerland), propranolol hydrochloride (Inderal: ICI, Macclesfield, Cheshire, U.K.). The concentrations given below are final molar concentration of the salt in the gel solution.

Statistics. Values are given as means $\pm$ s.e.m. Mean number of ruptured follicles in the ovaries from the various experimental groups were compared using Student's *t* test. Variance analysis was performed to evaluate differences in mean ovulation rates in some of the control groups. A *P* value < 0.05 was considered statistically significant.

Results

The schedule used (Text-fig. 1) resulted in a mean $\pm$ s.e.m. of 5.5 ± 0.6 ovulations per ovary, expressed as the number of corpora lutea counted 4 days after the gonadotrophin priming. Injection of the gel carrier into the ovarian bursa caused a slight, but not statistically significant, reduction in the number of corpora lutea (4.6 ± 0.7 per ovary).

The control groups of animals receiving gel carrier alone showed considerable variability in the mean values for the number of corpora lutea. This was confirmed by variance analysis of the 5 control groups of this type included in Table 1 ($F = 4.713$ at 4 and 42 degrees of freedom, giving $P < 0.01$). Therefore, comparison was made between simultaneously run control and experimental groups using the *t* test. It can be seen from Table 1 that noradrenaline and terbutaline administered into the ovarian bursa significantly increased the number of ovulations. In control experiments it was found that the same amount of these sympathomimetics injected systemically had no sig-

Table 1. Number of corpora lutea per ovary in PMSG-primed immature rats receiving bilateral injections in the ovarian bursa of active compounds carried in a gel, or gel solution alone (each ovary received 20 μ l of the gel, containing active substance in the molar concentration indicated)

Experimental treatment	No. of ovulations per ovary	Control treatment	No. of ovulations per ovary	% change in ovulation rate	Significance
Noradrenaline 10^{-5} M	4.8 ± 0.3 (18)	Gel carrier	3.8 ± 0.3 (13)	+29	$P < 0.05$
Phentolamine 10^{-5} M	3.7 ± 0.2 (13)	Gel carrier	5.0 ± 0.2 (12)	-27	$P < 0.001$
Noradrenaline 10^{-5} M	4.8 ± 0.2 (12)	Noradrenaline 10^{-5} M + phentolamine 10^{-4} M	4.2 ± 0.3 (15)	+13	N.S.
Isoprenaline 10^{-5} M	4.1 ± 0.5 (5)	Gel carrier	4.6 ± 0.8 (5)	-11	N.S.
Terbutaline 10^{-5} M	4.6 ± 0.2 (12)	Gel carrier	3.3 ± 0.3 (11)	+38	$P < 0.01$
Terbutaline 10^{-5} M	4.7 ± 0.3 (6)	Terbutaline 10^{-5} M + propranolol 10^{-4} M	3.0 ± 0.3 (8)	+56	$P < 0.01$
4-Aminopyridine 3×10^{-5} M	5.4 ± 0.4 (7)	Gel carrier	3.7 ± 0.3 (6)	+46	$P < 0.01$

Values are mean $\pm$ s.e.m. for the number of animals within parentheses. N.S. = non-significant.

nificant effect on the PMSG-induced ovulatory response, compared with animals receiving i.m. injection of gel carrier alone (4.4 ± 0.6 , 4.6 ± 0.5 and 4.9 ± 0.4 , respectively).

The local effect of terbutaline was counteracted by the β -receptor antagonist, propranolol (Table 1). On the other hand, the α -antagonist, phentolamine, was not able to block completely the noradrenaline-induced response, although the antagonist itself significantly reduced the frequency of ovulation. In contrast to the effect of the β_2 -selective agonist, terbutaline, there was no significant change in the number of corpora lutea in the presence of isoprenaline (Table 1).

Pharmacological activation of the autonomic nerve supply in the ovary by 4-aminopyridine also enhanced the number of corpora lutea significantly (Table 1).

Discussion

Priming of immature rats with PMSG provides a well characterized model for studies of the ovulation process. The treatment leads to follicular development, release of endogenous gonadotrophins and ovulation, with subsequent formation of corpora lutea (Herlitz *et al.*, 1976; Khan *et al.*, 1979; Ahrén *et al.*, 1981). The events leading to ovulation under these conditions are generally the same as in adult cyclic rats (Nuti *et al.*, 1975), but the lifespan of the corpus luteum in the PMSG-primed animals is prolonged as in pseudopregnant adult rats (Smith, Freeman & Neill, 1975; Taya & Greenwald, 1981).

The number of ovulations was estimated in terms of the number of corpora lutea present 4 days after the PMSG priming. This was preferred to counting of ova in the oviduct, since the transport mechanisms might have been disturbed during handling of the tubal tissues and ovarian adnexa in conjunction with the drug/gel injections. Indeed, the number of corpora lutea was slightly reduced, not statistically significantly so, by the injection itself into the ovarian bursa. Since immature rats were used, the corpora lutea formed upon gonadotrophin stimulation only represented one generation, which was easily recognized. There were no signs, by microscopic examination, of oocyte retention in the ovary and the number of corpora lutea was therefore considered to be a true reflection of the number of ovulations. Moreover, there was a complete agreement between the number of corpora lutea counted in the stereomicroscope and the number found by light microscopic examination of stained paraffin-wax sections. Cystic follicles were not detected, and characteristic corpora lutea were clearly distinguished from aberrant structures such as degenerating (atretic) follicles. The numbers obtained agree well with figures presented by Herlitz *et al.* (1976) using the same dose of PMSG in this type of animal model.

The experiments were carried out over an extended period of time. There was a statistically significant difference in the ovulation rate in the various control groups over this time period, as shown by analysis of variance. It was therefore considered most appropriate to compare the effects of the various active compounds with controls receiving gel carrier alone at the same time, as shown in Table 1. In the use of the *t* test it was assumed that both control and experimental responses were distributed with acceptable approximation to the normal distribution. A full analysis of variance would not lead to any further conclusions.

A cellulose gel with high viscosity was used as vehicle for the different test substances injected into the ovarian bursa, to avoid leakage and to provide high local concentrations without systemic effects. It can be assumed that the effect of the substance is prolonged due to slow release, and an active residue may even be present in the ovarian bursa at the time of ovulation. The absence of systemic actions on the ovulatory process of the small amount injected locally into the ovarian bursa was checked with noradrenaline and terbutaline. Thus, when doses of these compounds equal to those administered into the ovarian bursa were injected i.m., they had no effect on the number of corpora lutea.

Noradrenaline caused a significant enhancement in the number of ovulations after PMSG priming. This agrees with the potentiating action of the neurotransmitter on ovulations induced *in vitro* by human chorionic gonadotrophin (hCG) (Kobayashi *et al.*, 1983). It is possible that the effect is

caused by contraction of follicular smooth muscle cells mediated by α -adrenergic receptors (Wallis, Edvinsson, Owman, Sjöberg & Svensson, 1975). Stimulation of these receptors has been shown to increase the intrafollicular pressure (Owman, Sjöberg, Svensson & Wallis, 1975). The effect of noradrenaline was not completely inhibited by the α -receptor antagonist, phentolamine. The dose of phentolamine may have been too low, or the receptor may have been insufficiently blocked under conditions when both agonist and antagonist were administered simultaneously. The remaining ovulatory response could also be due to the β -agonistic action of noradrenaline not affected by phentolamine. It was notable that phentolamine alone diminished the number of ovulations. Similar observations have been made with ovaries receiving the antagonist into the aorta close to the origin of the ovarian artery (Virutamasen, Hickok & Wallach, 1971) or with ovaries stimulated with hCG and perfused *in vitro* (G. Schmidt, Ch. Owman, N.-O. Sjöberg & B. Wallis, unpublished). It may reflect the presence of a sympathetic tone in the follicle wall, resulting in an α -receptor mediated contraction. This agrees with the observation that noradrenaline, released from sympathetic nerve terminals in the theca externa, is able to contract its smooth musculature (Wallis *et al.*, 1982). Also, the irreversible α -receptor antagonist, phenoxybenzamine, has been found to reduce the number of corpora lutea in PMSG-primed rats (Wallis *et al.*, 1977a).

The β_2 -receptor agonist terbutaline, like noradrenaline, increased the incidence of ovulations. Again, this corroborates observations of ruptured follicles made on the rabbit ovary perfused *in vitro* (G. Schmidt, Ch. Owman, N.-O. Sjöberg & B. Wallis, unpublished). The effect was counteracted by the antagonist, propranolol. However, it could not be reproduced with the non-selective β -agonist, isoprenaline, which might indicate that selective stimulation of the β_2 type of receptor is required for the response.

It has been shown that β_2 types of adrenergic receptors mediate relaxation in isolated strips of human Graafian follicles (Wallis, Falck, Owman & Sjöberg, 1977b), which would perhaps be expected to cause a reduced rate of ovulation. However, stimulation of ovarian β_2 -receptors increases the formation of cyclic 3,5-adenosine monophosphate (cAMP), which is linked to an activated synthesis of progesterone *in vitro*, both in follicular and luteal tissue (Ratner, Weiss & Sanborn, 1980; Norjavaara, Selstam & Ahrén, 1982; Zsolnai, Varga & Horvath, 1982). Accordingly, terbutaline, but not the β_1 -selective agonist dobutamine, stimulates progesterone production in granulosa cells of FSH-primed rats (Adashi & Hsueh, 1981). Progesterone, in turn, appears to stimulate the frequency of ovulations *in vitro* (Baranczuk & Fainstat, 1976; Hamada, Wright & Wallach, 1979; Testart, Thébault & Lefèvre, 1983) and *in vivo* (Takahashi, Ford, Yoshinaga & Greep, 1974). This would suggest that the effect of terbutaline is mediated by a local humoral action rather than by a mechanical influence on the follicle wall, unless the responsiveness of its smooth muscle cells has been altered by the presence of progesterone.

Since the smooth musculature in the walls of the ovarian blood vessels is also influenced by β -agonists (Varga, Zsolnai & Bernard, 1979), the flow through the ovarian tissue should be increased in the presence of terbutaline due to vasodilatation. It has been shown that the intrafollicular pressure is influenced by the ovarian blood flow (Lipner & Smith, 1971), and the net effect of a β -receptor stimulation might thus be an increased intrafollicular pressure. This could be another factor promoting an increased incidence of ovulations.

The experiments indicate that both α - and β -adrenergic receptors mediate an increased rate of ovulation through an effect, on the one hand, at the level of the follicle wall and, on the other hand, by a humoral-type of ovarian mechanism. Attempts were made with 4-aminopyridine to increase the efficiency by which noradrenaline is released during adrenergic nerve activation (Glover, 1982). In accordance with the findings following local administration of the sympathomimetic stimulants, this agent markedly enhanced the incidence of ovulations after PMSG priming.

The study was supported by grants from the Swedish Medical Research Council (No. 14X-5680) and AB Draco, Lund (Sweden). We thank Ms Mary-Ann Sällström for skilful technical assistance and Associate Professor Claus Rerup for expert statistical advice.

References

- Adashi, E.Y. & Hsueh, A.J.W. (1981) Stimulation of β_2 -adrenergic responsiveness by follicle stimulating hormone in rat granulosa cells *in vitro* and *in vivo*. *Endocrinology* **108**, 2170–2178.
- Ahrén, K., Khan, I. & Selstam, G. (1981) Different patterns of cyclic AMP accumulation in rat corpora lutea after LH stimulation *in vitro* and *in vivo*. *J. Reprod. Fert.* **61**, 157–162.
- Amsterdam, A., Lindner, H.R. & Gröschel-Stewart, U. (1977) Localization of actin and myosin in the rat oocyte and follicular wall by immunofluorescence. *Anat. Rec.* **187**, 311–327.
- Baranczuk, R.J. & Fainstat, T. (1976) In-vitro ovulation from adult hamster ovary. *Am. J. Obstet. Gynec.* **124**, 517–522.
- Burden, H.W. (1978) Ovarian innervation. In *The Vertebrate Ovary*, pp. 615–638. Ed. R. E. Jones. Plenum Press, New York.
- Glover, W.E. (1982) The aminopyridines: a review. *Gen. Pharmac.* **13**, 259–285.
- Hamada, Y., Wright, K.H. & Wallach, E.E. (1979) The effects of progesterone and human chorionic gonadotrophin on ovulation in the *in vitro* perfused rabbit ovary. *Fert. Steril.* **32**, 335–339.
- Herlitz, H., Koch, Y., Khan, I. & Ahrén, K. (1976) Effects of follicle stimulating hormone on cyclic AMP levels in young corpora lutea of the rat. *Eur. J. Obstet. Gynec. Reprod. Biol.* **6**, 175–179.
- Khan, I.M., Rosberg, S., Lahav, M., Lamprecht, S.A., Selstam, G., Herlitz, H. & Ahrén, K. (1979) Studies on the mechanism of action of the inhibitory effect of prostaglandin $F_{2\alpha}$ on cyclic AMP accumulation in rat corpora lutea of various ages. *Biol. Reprod.* **21**, 1175–1183.
- Kobayashi, Y., Sjöberg, N.-O., Walles, B., Owman, Ch., Wright, K.H., Santulli, R. & Wallach, E.E. (1983) The effects of adrenergic agents on the ovulatory process in the *in vitro* perfused rabbit ovary. *Am. J. Obstet. Gynec.* **145**, 857–864.
- Lipner, H. & Smith, M.S. (1971) A method for determining the distribution and source of protein in preovulatory rat ovaries. *J. Endocr.* **50**, 187–200.
- Mohsin, S. & Pennefather, J.N. (1979) The sympathetic innervation of the mammalian ovary. A review of pharmacological and histological studies. *Clin. Exp. Pharmacol. Physiol.* **6**, 335–354.
- Norjavaara, E., Selstam, G. & Ahrén, K. (1982) Catecholamine stimulation of cyclic AMP and progesterone production in rat corpora lutea of different ages. *Acta endocr., Copenh.* **100**, 613–622.
- Nuti, K.M., Sridharan, B.N. & Meyer, R.K. (1975) Reproductive biology of PMSG-primed immature female rats. *Biol. Reprod.* **13**, 38–44.
- Owman, Ch., Sjöberg, N.-O., Svensson, K.-G. & Walles, B. (1975) Autonomic nerves mediating contractility in the human Graafian follicle. *J. Reprod. Fert.* **45**, 553–556.
- Owman, Ch., Sjöberg, N.-O., Wallach, E.E., Walles, B. & Wright, K.H. (1979) Neuromuscular mechanisms of ovulation. In *Human Ovulation*, pp. 57–100. Ed. E. S. E. Hafez. Elsevier/North Holland Biomedical Press, Amsterdam.
- Ratner, A., Weiss, G.K. & Sanborn, C.R. (1980) Stimulation by β_2 -adrenergic receptors of the production of cyclic AMP and progesterone in rat ovarian tissue. *J. Endocr.* **87**, 123–129.
- Smith, M.S., Freeman, M.E. & Neill, J.D. (1975) The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotrophin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology* **96**, 219–226.
- Stefenson, A., Owman, Ch., Sjöberg, N.-O., Sporrang, B. & Walles, B. (1981) Comparative study of the autonomic innervation of the mammalian ovary, with particular regard to the follicular system. *Cell Tissue Res.* **215**, 47–62.
- Taya, K. & Greenwald, G.S. (1981) Post-ovulatory steroidogenesis after PMS-induced ovulation in immature female rats. *Am. J. Physiol.* **241**, E221–E225.
- Takahashi, M., Ford, I., Yoshinaga, K. & Greep, R.O. (1974) Induction of ovulation in hypophysectomized rats by progesterone. *Endocrinology* **95**, 1322–1326.
- Testart, J., Thébault, A. & Lefèvre, B. (1983) In-vitro ovulation of rabbit ovarian follicles isolated after the endogenous gonadotrophin surge. *J. Reprod. Fert.* **68**, 413–418.
- Varga, B., Zsolnai, B. & Bernard, A. (1979) Stimulation of alpha- and beta-adrenergic receptors in human ovarian vasculature *in vitro*. *Gynecol. Obstet. Invest.* **10**, 81–87.
- Virutamasen, P., Hickok, R.L. & Wallach, E.E. (1971) Local ovarian effects of catecholamines on human chorionic gonadotrophin-induced ovulation in the rabbit. *Fert. Steril.* **22**, 235–243.
- Walles, B., Edvinsson, L., Owman, Ch., Sjöberg, N.-O. & Svensson, K.-G. (1975) Mechanical response in the wall of ovarian follicles mediated by adrenergic receptors. *J. Pharmac. exp. Ther.* **193**, 460–473.
- Walles, B., Edvinsson, L., Owman, Ch., Sjöberg, N.-O., Sporrang, B. & Stefenson, A. (1977a) Influence of sympathetic nerves, amine receptors and anti-adrenergic drugs on follicular contractility and ovulation. *Acta physiol. scand., Suppl.* **452**, 113–120.
- Walles, B., Falck, B., Owman, Ch. & Sjöberg, N.-O. (1977b) Characterization of autonomic receptors in the smooth musculature of human Graafian follicle. *Biol. Reprod.* **17**, 423–431.
- Walles, B., Gröschel-Stewart, U., Owman, Ch., Sjöberg, N.-O. & Unsicker, K. (1978) Fluorescence histochemical demonstration of a relationship between adrenergic nerves and cells containing actin and myosin in the rat ovary, with special regard to the follicle wall. *J. Reprod. Fert.* **52**, 175–178.
- Walles, B., Owman, Ch. & Sjöberg, N.-O. (1982) Contraction of the ovarian follicle induced by local stimulation of its sympathetic nerves. *Brain Res. Bull.* **9**, 757–760.
- Zsolnai, B., Varga, B. & Horvath, E. (1982) Increase of ovarian progesterone secretion by β_2 -adrenergic stimulation in oestrous rats. *Acta endocr., Copenh.* **101**, 268–272.

Received 22 November 1984