Abstract. The biosynthesis of polyamines is dramatically increased in the ovaries of rat and hamster during the evening of pro-oestrus. In an attempt to shed some light on the physiological function of this biosynthesis ornithine decarboxylase (ODC), which catalyzes the rate-limiting step in the biosynthesis of the polyamines, was immunohistochemically localized in the ovaries from rat and hamster during pro-oestrus. At dioestrus, only a few immunoreactive cells were found in the ovaries. During the evening of pro-oestrus, on the other hand, numerous immunoreactive cells were observed in the ovaries. These cells were confined to the internal thecal layer of Graafian as well as smaller follicles and to the interstitial tissue of the ovary. The granulosa cells appeared to be devoid of immunoreactive ODC. The hamster ovary, which during this time exhibited considerably higher levels of ODC activity than the ovaries from the rat, did accordingly contain more immunoreactive cells than the rat ovary.

The cellular effects of several steroid and polypeptide hormones include an induction of the enzyme ornithine decarboxylase (ODC). ODC catalyzes the rate-limiting step in the biosynthesis of the polyamines putrescine, spermidine and spermine. The specific function of these amines is unknown, although results from numerous studies indicate a close correlation between polyamines and cellular growth (for reviews, see Heby & Jänne 1981; Pegg & McCann 1982; Tabor & Tabor 1984).

During the oestrous cycle of the rat and the hamster, a large and transitory rise in ODC activity is observed in the ovaries on the evening of pro-oestrus (Kobayashi et al. 1971; Sheela Rani & Moudgal 1979). The induction of ovarian polyamine synthesis appears to be caused by the preovulatory surge of luteinizing hormone (LH). Administration of LH or human chorionic gonadotropin (hCG) causes a marked increase in ODC activity in the ovaries of immature as well as cycling rats (Kobayashi et al. 1971; Williams-Asman et al. 1972; Kaye et al. 1973; Maudsley & Kobayashi 1974). Furthermore, antiserum against LH given during the afternoon of pro-oestrus totally abolishes the transitory rise in ovarian ODC activity (Kobayashi et al. 1971). Follicle stimulating hormone (FSH) has only minor effects on ovarian polyamine biosynthesis when given in vivo (Kobayashi et al. 1971; Sheela Rani & Moudgal 1979). In vitro, however, FSH is a potent inducer of ODC activity in porcine granulosa cells (Osterman & Hammond 1977).

The specific timing of the polyamine synthesis in the ovaries during the oestrous cycle has raised speculations upon an essential role of these amines in the ovulatory process. This belief is strengthened by the finding that a specific irreversible inhibitor of ODC, \(\alpha\)-difluoromethylornithine, enhances ovulation when given to the rats at pro-oestrus (Fozard et al. 1980; Carpenter & Fozard 1982).

To unravel the physiological function of the polyamines synthesized during pro-oestrus, identification of the specific parts of the ovaries that are involved in this biosynthesis appears crucial. However, information is scarce. By surgical isolation Iceksion et al. (1974) found a preferential increase in ODC activity in the Graafian follicles.
of the pro-oestrus rat ovary. In the hamster, on the other hand, the increase in ovarian ODC activity during pro-oestrus seems to be associated with both the Graafian follicles and the remaining ovarian tissue (Sheela Rani & Moudgal 1979).

The distribution of ODC has been studied in greater detail in ovaries from prepubertal rats after administration of hCG (Persson et al. 1982a). In these rats, ODC was immunohistochemically shown to be confined to the thecal layer of the follicles and to the interstitial tissue of the ovaries. In the present report, immunohistochemical techniques have been employed in an attempt to localize the polyamine biosynthesis in ovaries from the mature rat and hamster during pro-oestrus.

Material and Methods

**Animals**

Adult female Sprague-Dawley rats, body weight 250–350 g, and adult female golden hamsters, body weight 120–160 g, were used. The animals were kept under controlled illumination (500 lux, lights on from 05.00 h to 19.00 h) and had free access to food and water. Timing of the oestrous cycle was determined by examination of daily vaginal smears and only animals which had showed two or more regular cycles were selected for use. In the hamster, the day of oestrus was characterized also by a thick yellowish odorous vaginal discharge.

**Assay of ODC activity**

Ovaries were homogenized in 1.2 ml of cold 0.1 mol/l Tris-HCl, pH 7.5, containing 2.5 mmol/l dithiothreitol and 0.1 mmol/l EDTA. After centrifugation at 20,000 × g for 20 min at 4°C, the ODC activity was assayed in an aliquot of the supernatant by measuring the release of 14CO2 from carboxyl-labelled ornithine. Incubations were performed in the presence of 0.1 mol/l Tris-HCl, pH 7.5, 2.5 mmol/l dithiothreitol, 0.1 mmol/l EDTA, 0.1 mmol/l pyridoxal 5’-phosphate and 0.5 mmol/l L-14C-ornithine (specific activity 2 mCi/mmol). The reaction was terminated by adding 0.3 ml of 2 mol/l perchloric acid. The expelled CO2 was trapped in a polypropylene centre well containing 100 µl of hydroxide of Hyamine 10-X (Packard Instrument AB, Stockholm, Sweden). Maximal absorption of CO2 was achieved by continuous shaking for additional 45 min. The centre well was then placed in 8 ml of Lipoluma scintillation mixture (LKB AB, Stockholm, Sweden), and the radioactivity was measured in a liquid scintillation spectrometer. All values obtained were corrected against a reaction mixture without enzyme. Assay variation (expressed as the per cent coefficient of variation) was less than 5%. One unit of enzyme activity was defined as the amount releasing 1 nmol CO2 per h.

**Immunotitration of ODC from hamster ovary**

The cross-reactivity of an antiserum against ODC from mouse kidney (Persson 1982) with ODC from hamster ovary was examined by immunotitration. Aliquots of the 20,000 × g supernatant (about 3.3 units of ODC activity) was incubated overnight at 4°C with different amounts of the ODC antiserum in 0.4 ml 0.1 mol/l Tris-HCl, pH 7.5, 0.1 mmol/l EDTA, and 2.5 mmol/l dithiothreitol containing 0.2% bovine serum albumin. To precipitate the antigen-antibody complexes, 25 µl of protein A adsorbent was added and incubated for an additional 90 min at 4°C. The incubate was centrifuged at 12,000 × g for 2 min, and ODC activity was determined in aliquots of the supernatant. Assay variation (expressed as the per cent coefficient of variation) was less than 5%.

**Immunohistochemistry**

Specimens from the ovaries were fixed overnight in cold formaldehyde solution (4% formaldehyde in 0.1 mol/l phosphate buffer, pH 7.2). After thorough rinsing in phosphate buffer containing 5% sucrose, the specimens were frozen on dry ice and sectioned on a cryostat at 10–15 µm. The sections were then processed for immunohistochemical demonstration of ODC using the antiserum against mouse kidney ODC (Persson 1982). The site of the antigen-antibody reaction was revealed by the indirect immunofluorescence technique of Coons et al. (1955) or by the peroxidase-antiperoxidase (PAP) method (Sternberger 1979).

In the immunofluorescence procedure, sections were incubated for 3 h at room temperature with ODC antiserum, diluted 1:80 with phosphate buffered saline (PBS) containing 0.25% bovine serum albumin and 0.25% Triton X-100. Thereafter the sections were thoroughly rinsed in PBS containing 0.25% Triton X-100, and the immunoreaction was visualized by incubation for 30 min with fluorescein-conjugated goat anti-rabbit IgG diluted 1:20. The sections were then rinsed in PBS containing 0.25% Triton X-100 and mounted in phosphate-buffered glycerin (PBS and glycerin 1:1). The sections were examined in a fluorescence microscope equipped with filters selected to give peak excitation at 490 nm.

In the PAP procedure, sections were incubated for 18 h at 4°C with the ODC antiserum, diluted 1:320. After washing in PBS containing 0.25% Triton X-100 the sections were incubated for 30 min at room temperature with sheep anti-rabbit IgG diluted 1:30. The sections were then rinsed and incubated for 30 min at room temperature with rabbit peroxidase-antiperoxidase complex diluted 1:160. After rinsing in Tris buffer, pH 7.6, the peroxidase activity was visualized by
Ornithine decarboxylase activity in ovaries of rat (A) and hamster (B) during the oestrous cycle. Values are means ± SEM (N = 2–12). D: dioestrus; P: pro-oestrus; O: oestrus; M: metoestrus.

Immunotitration of ornithine decarboxylase from hamster ovary. Increasing amounts of control (○) or anti-ornithine decarboxylase (●) serum were added to a fixed quantity of enzyme activity. After incubation and precipitation as described in Material and Methods remaining enzyme activity was determined. Values are means ± SEM (N = 4).
incubation with a solution of 0.06% 3,3-diaminobenzenidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 mol/l Tris buffer, pH 7.6, for 1 h at room temperature. The sections were then rinsed in water, dehydrated in a series of ethanol solutions and xylene, and finally mounted in Permount (Fisher Scientific Co, Fair Lawn, NJ), and examined using an ordinary light microscope.

Determination of protein
Protein in supernatants was measured by the method of Lowry et al. (1951), with bovine serum albumin as standard.

Results

ODC activity in the ovaries from rat and hamster during the oestrous cycle
ODC activity was measured in ovaries from rats and hamsters at different stages of the oestrous cycle. The results are given in Fig. 1. Both species exhibited a large transitory rise in ovarian ODC activity during the afternoon of pro-oestrus. In the rat, ovary peak values of ODC activity were observed on pro-oestrus between 19.00 h and 21.00 h, whereas in the hamster the peak occurred a little earlier, namely between 16.00 h and 20.00 h (Fig. 1A and B). The induction of ODC activity in the ovaries during pro-oestrus was stronger in the hamster than in the rat, reaching values of about 50 units per mg protein as compared with peak values of approximately 12 units per mg protein in the rat ovary.

Immunotitration of hamster ovary ODC with an antiserum against mouse kidney ODC
A very potent and monospecific antiserum against ODC has been raised in a rabbit using homogeneous preparations of ODC from kidneys of testosterone-treated mice (Persson 1982). This antiserum has been shown effectively to cross-react with ornithine decarboxylase from different rat tissues, including the ovaries (Persson 1982). The antiserum has furthermore been used immunohistochemically to localize ODC in various tissues of the mouse and rat (Persson et al. 1982a,b, 1983a,b). In the present study, the cross-reactivity of this antiserum against hamster ovary ODC was studied by immunotitration. As shown in Fig. 2, the antiserum was found effectively to precipitate ODC from hamster ovary. The amount of antiserum required to precipitate 50% of 1 unit of enzyme activity was calculated to be about 1.1 nl. This value is quite similar to those earlier obtained against ODC from different rat and mouse tissues (0.8–1.1 nl) (Persson 1982), indicating a close, if not identical, immunological relationship between ODC from mouse, rat and hamster. Hence, it appears that the antiserum against mouse kidney ODC could be useful for immunohistochemical localization of ODC also in the hamster ovary.

Immunohistochemical localization of ODC in the ovaries from rat and hamster at pro-oestrus
Ovaries taken from rats at 19.00 h and from hamsters at 18.00 h during dioestrus and pro-oestrus were processed for ODC immunohistochemistry (Fig. 3). At dioestrus, the ovaries did not display any significant amount of immunoreactive ODC, either in the rat or in the hamster. Only a few immunoreactive cells were found scattered in the ovaries at this stage of the oestrous cycle. During the evening of pro-oestrus, on the other hand, numerous immunoreactive cells were observed in the ovaries. In the rat, they were confined to the internal thecal layer of some of the follicles as well as to small parts of the interstitial tissue. The granulosa cells appeared to be devoid of immunoreactive ODC. Immunoreactive theca cells were found not only in the Graafian follicles but also smaller follicles were shown to contain immunoreactive ODC in their surrounding thecal layer.

The hamster ovaries, which during the evening of pro-oestrus exhibited considerably higher levels of ODC activity than those from the rat, accordingly contained substantially more immunoreactive cells. As in the rat, these cells were mainly found in the thecal layer of the follicles and in the interstitial part of the ovary. Most of the follicles as well as the major part of the interstitial tissue exhibited immunoreactive ODC. Hence, immunoreactivity in the thecal layer was not restricted to the Graafian follicles. The granulosa cells were often devoid of immunoreactive ornithine decarboxylase. However, a few of the follicles contained immunoreactive granulosa cells. The amount of immunoreactivity found in these cells was, nevertheless, considerably smaller than in the theca cells or in the cells of the interstitial tissue.

In both rats and hamsters, the immunoreactivity appeared to be mainly, if not exclusively, located in the cytoplasmic part of the cells.
Immunohistochemical localization of ornithine decarboxylase in ovaries of rat and hamster.

a) Section of a rat ovary taken at 19.00 h on pro-oestrus, showing immunofluorescence in the thecal cells and in the interstitial tissue. Bar = 50 µm.

b) Section of a hamster ovary taken at 18.00 h on pro-oestrus and stained with the PAP technique, demonstrating strongly stained interstitial tissue as well as several small follicles with unstained granulosa cells. Bar = 200 µm.

c) Section of a hamster ovary taken at 18.00 h on pro-oestrus, demonstrating intensely immunofluorescent cells in the interstitial tissue as well as in the thecal layer. Bar = 50 µm.

d) Section of a hamster ovary taken at 18.00 h on dioestrus, showing few weakly immunofluorescent cells. Bar = 50 µm.
Discussion

The induction of ODC activity in the rat and hamster ovaries during the evening of pro-oestrus is one of the strongest inductions of this enzyme ever reported, reaching activity values of up to 20 times those usually found in other tissues when ODC is fully induced. The increase, however, was more conspicuous in the hamster than in the rat, which probably reflects a difference in sensitivity to LH, since the pre-ovulatory release of LH is about the same in both species. The physiological function of this biosynthesis of polyamines during pro-oestrus is still unknown. It has been suggested that the polyamines are fulfilling some important role associated with the ovulatory process occurring soon after (Kobayashi et al. 1971). Such a function would also suggest that the biosynthesis of polyamines is taking place in close connection with the Graafian follicles. This has in fact been demonstrated by Icekson et al. (1974), who surgically isolated and measured ODC activity in the Graafian follicles and corpora lutea from ovaries of pro-oestrus rats treated with LH. Ornithine decarboxylase activity was found to be preferentially enhanced in the Graafian follicles (Icekson et al. 1974). However, the remaining ovarian tissue was not examined for ODC activity and, hence, no information was obtained as to ODC activity being exclusively confined to the Graafian follicles.

As shown in the present report ODC did not appear to be restricted only to the Graafian follicles in the rat ovary at the pro-oestrus stage of the cycle. Indeed, immunoreactive ODC was also found in the thecal layer of some of the smaller follicles as well as in parts of the interstitial tissue. Using a similar technique as Icekson et al. (1974), Sheela Rani & Moudgal (1979) reported that the increase in ODC activity in the hamster ovary during late pro-oestrus is associated with both the Graafian follicles and the rest of the ovarian tissue, which chiefly consists of interstitium, stroma, and growing follicles. The present study revealed that the induction of ODC takes place in the thecal layer of as well the Graafian as smaller follicles and in the interstitial part of the ovary.

Hence, it appears that in both rats and hamsters, ODC was induced in the same ovarian structures during the evening of pro-oestrus. In the rat ovary, however, only a few of the follicles and only parts of the interstitium showed immunoreactivity, whereas in the hamster ovary most of the follicles and the interstitium displayed immunoreactive ODC. This is probably due to the fact that the induction of ODC during pro-oestrus was considerably larger in the hamster than in the rat ovary. The immunohistochemical technique is only semi-quantitative and, thus, what showed up to be positive in the rat ovary were only the parts which contained the highest amounts of ODC, i.e. the tip of the iceberg effect. The same is also true for the granulosa cells. A few follicles in the hamster ovary contained granulosa cells displaying immunoreactivity, indicating that ODC was also induced in these cells during pro-oestrus. Nevertheless, it is quite clear that the major induction of ODC in the ovaries during pro-oestrus was confined to the thecal layer of the follicles, both Graafian and smaller ones, as well as to the interstitial cells of the ovary. This distribution of ovarian ODC is similar to that found in the ovaries of immature rats treated with hCG (Persson et al. 1982a).

The localization of ODC to other parts than the Graafian follicles suggests that the polyamines also could be involved in other processes than the ovulation, e.g. initiation of follicular growth, which occurs during this stage of the oestrous cycle (Moore & Greenwald 1974; Sheela Rani & Moudgal 1977; Richards 1980). This is also indicated by the finding that treatment with the ODC inhibitor, α-difluorornithine, during pro-oestrus not only resulted in an increased ovulation the next day but also gave rise to an increased number of eggs released during the subsequent cycle (Carpenter & Fozard 1982). Alternatively, the polyamines may fulfil other functions in connection with the enhanced metabolic activity, i.e. steroid hormone synthesis, and the hypertrophic growth of the thecal and interstitial cells evoked by the pre-ovulatory surge of gonadotropins (Guraya 1978).

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References


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