Changes in the density of peripheral benzodiazepine binding sites in genital organs of the female rat during the oestrous cycle*

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Summary. The affinity and the density of peripheral-type benzodiazepine binding sites (PBzS) in tissues from the genital organs of female rats were studied during the oestrous cycle. When comparing PBzS density on the day of oestrus to PBzS density on the day of pro-oestrus, a significant increase was observed in the ovary (1.9-fold), oviduct (2.4-fold) and uterus (1.7-fold), but not in the kidney. Serum oestradiol also increased to a maximum on the day of pro-oestrus. The ovarian and uterine PBzS density and serum concentrations of oestradiol and progesterone were measured every 8 h between the days of dioestrus and pro-oestrus. Ovarian and uterine PBzS density increased to a maximal value at 01:00 and 09:00 h, respectively, on the day of pro-oestrus. However, a significant increase in PBzS density occurred in the ovary ($P < 0.02$) and uterus ($P < 0.001$) at 09:00 h on the day of pro-oestrus as compared to 09:00 h on the day of dioestrus. These changes were associated with an increase in serum oestradiol and progesterone concentrations. The affinity of PBzS in all tissues examined remained unaltered during the oestrous cycle. This study demonstrates that changes associated with the oestrous cycle occur in the density of PBzS in various genital organs.

Keywords: peripheral benzodiazepine binding sites; ovary; oviduct; uterus; oestrous cycle; rat

Introduction

Benzodiazepines are used clinically as anxiolytics, hypnotics, anticonvulsants and muscle relaxants. It has been suggested that the effect of benzodiazepines in the central nervous system is mediated by specific receptors which are coupled with the γ -aminobutyric acid (GABA) receptors (Tallman *et al.*, 1978).

Peripheral-type benzodiazepine binding sites (PBzS) have been identified in peripheral tissues such as lung, liver and kidney (Braestrup & Squires, 1977), mast cells (Taniguchi *et al.*, 1980), platelets (Wang *et al.*, 1980) and uterus and oviduct (Fares *et al.*, 1987). PBzS differ from central-type benzodiazepine receptors in their lack of coupling to GABA receptors and in their ligand specificity (Marangos *et al.*, 1982): Ro 5-4864 (a benzodiazepine) and PK 11195 (an isoquinoline carboxamide derivative) bind with high affinity to PBzS, but not to central-type benzodiazepine receptors. The reverse is true with regard to specific central-type ligands such as clonazepam.

PBzS are coupled to calcium channels (Mestre *et al.*, 1985). It has also been shown that a PBzS selective ligand, Ro 5-4864, blocks calcium channels in the heart, whereas PK 11195 reverses the

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effect of Ro 5-4864 and even the effect of other known calcium ion channel blockers such as nitrendipine, nifedipine and verapamil (Mestre *et al.*, 1985).

PBzS have also been characterized in several endocrine organs such as the hypophysis (De Souza *et al.*, 1985; Voigt *et al.*, 1984), adrenal, testis (De Souza *et al.*, 1985), term placenta (Fares & Gavish, 1985) and ovary (Fares *et al.*, 1987). Several studies indicate that PBzS density in the endocrine organs is affected by their trophic hormones: hypophysectomy reduced PBzS density in the testis and adrenal (Anholt *et al.*, 1985); chronic treatment with oestrogen in adult male rats also reduced PBzS density in the testis (Gavish *et al.*, 1986); and treatment with oestrogen or pregnant mares' serum gonadotrophin (PMSG) increased PBzS density in rat ovary, oviduct and uterus, but not in kidney (Fares *et al.*, 1987). It has been suggested that the effect of PMSG on the ovary, oviduct and uterus is mediated by ovarian oestrogen synthesis (Fares *et al.*, 1987).

The present study was therefore undertaken to examine the relationship between the cyclic changes in endogenous plasma oestradiol and progesterone concentrations during the rat oestrous cycle and PBzS density in the ovary, oviduct and uterus.

Materials and Methods

Ligands. [^3H]PK 11195 (sp. act. 74.5 Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. Unlabelled Ro 5-4864 was kindly supplied by Dr H. Gutmann and Dr E. Kyburz, Hoffmann-La Roche, Basel, Switzerland. Lumax was purchased from Lumac, Schaesburg, The Netherlands.

Animals. Female Sprague-Dawley rats (180–200 g) were housed in air-conditioned quarters with a 12-h light/dark schedule (lights on between 07:00 and 19:00 h). Standard rat pellets and tap water were provided *ad libitum*. Vaginal smears were examined every morning between 08:00 and 10:00 h. Only rats which had at least two successive regular 4-day cycles immediately before the experiment were used. Within 1 h following the last vaginal-smear test, rats were killed by decapitation, and blood was collected immediately. Blood was left at room temperature for 1 h, and then serum was separated by centrifugation at 200 *g* for 20 min and stored at -20°C until assayed for oestradiol and progesterone. The kidneys, ovaries, oviducts and uteri were removed, trimmed of adherent tissues, washed in saline and stored at -20°C .

Preparation of membranes. Tissues were homogenized in 50 volumes of 50 mM ice-cold Tris-HCl buffer, pH 7.4, using a Brinkmann Polytron (setting 10) for 15 sec. The homogenate was centrifuged at 49 000 *g* for 15 min. The pellet was suspended in 50 mM ice-cold Tris-HCl buffer, pH 7.4, to achieve about 0.25–0.62 mg protein/ml and used for binding assay.

[^3H]PK 11195 binding assay. Membranes (400 μl , 0.10–0.25 mg protein) were incubated with 25 μl [^3H]PK 11195 (0.18–6 nM, final concentration) in the absence (total binding) or presence (non-specific binding) of 75 μl unlabelled Ro 5-4864 (1 μM , final concentration) in 500 μl final volume. After incubation for 60 min at 4°C , samples were filtered under vacuum over Whatman GF/B filters and washed three times with 5 ml 50 mM ice-cold Tris-HCl buffer. Filters were placed in vials containing 5 ml Xylene-Lumax (3:1 v/v) and counted for radioactivity. Protein content was determined as described by Lowry *et al.* (1951).

Assays for serum oestradiol and progesterone concentrations. Oestradiol was measured by radioimmunoassay by using a kit from Isodan Diagnostic Laboratories, Jerusalem, Israel, with ^{125}I -labelled oestradiol as the tracer hormone. The antibodies directed against oestradiol are very specific and have very low cross-reactivity with other steroids measured at 50% displacement compared to oestradiol. For the various tested androgens the cross-reactivity did not exceed 0.005%; for oestriol and oestrone it was $<0.5\%$ and $<11.5\%$, respectively, and for progesterone it was $<0.001\%$. Progesterone was measured by RIA using a kit from Diagnostic Products Corporation, Los Angeles, CA, U.S.A., with ^{125}I -labelled progesterone as the tracer hormone. The antibodies directed against progesterone are very specific and have very low cross-reactivity with other steroids: androgens, not detectable; corticosterone, 0.4%; oestradiol, not detectable; and 20α -dihydroprogesterone, 2.0%. The sensitivities of the assays for oestradiol and progesterone were 5 pg/ml and 0.05 ng/ml, respectively.

Statistical analyses. The statistical significances were determined by one-way analysis of variance (ANOVA) or Student's *t* test.

Results

The mean maximal number of binding sites and equilibrium dissociation constant of [^3H]PK 11195 binding in the ovary, oviduct and uterus of mature female rats were measured throughout the 4

days of the oestrous cycle. When comparing PBzS density on the day of oestrus to PBzS density on the day of pro-oestrus, a significant increase ($P < 0.01$) was observed in the ovary (1.9-fold), oviduct (2.4-fold) and uterus (1.7-fold), but not in the kidney (Table 1). When comparing PBzS density in the ovary, oviduct and uterus on days of oestrus, metoestrus and dioestrus, no significant change ($P > 0.05$) was observed. The increase in PBzS density in the ovary, oviduct and uterus on the day of pro-oestrus was not accompanied by a change in PBzS affinity (Table 1). Serum oestradiol concentrations were 34 ± 8 , 32 ± 17 , 39 ± 6 and 92 ± 11 pg/ml on the days of oestrus, metoestrus, dioestrus and pro-oestrus, respectively (7 rats/day). The 2.3-fold increase in oestradiol concentration on the day of pro-oestrus was positively correlated with the increase in PBzS density in the ovary, oviduct and uterus.

Table 1. Maximal number of binding sites (fmol/mg protein) and equilibrium dissociation constants (nM, in parentheses) of [3 H]PK 11195 binding to the genital organs and kidney of adult female rats throughout the 4-day oestrous cycle

Organ	Day of cycle			
	Oestrus	Metoestrus	Dioestrus	Pro-oestrus
Ovary	1888 $\pm$ 141 (1.4 $\pm$ 0.2)	2138 $\pm$ 290 (1.5 $\pm$ 0.3)	2459 $\pm$ 272 (1.5 $\pm$ 0.4)	3580 $\pm$ 204* (1.6 $\pm$ 0.2)
Oviduct	1319 $\pm$ 150 (1.1 $\pm$ 0.1)	1563 $\pm$ 118 (1.6 $\pm$ 0.4)	1039 $\pm$ 207 (1.5 $\pm$ 0.3)	3236 $\pm$ 320* (1.4 $\pm$ 0.3)
Uterus	2528 $\pm$ 310 (1.8 $\pm$ 0.3)	1770 $\pm$ 292 (1.3 $\pm$ 0.2)	2076 $\pm$ 212 (1.4 $\pm$ 0.1)	4266 $\pm$ 282* (1.6 $\pm$ 0.3)
Kidney	3810 $\pm$ 338 (1.8 $\pm$ 0.2)	4247 $\pm$ 346 (1.6 $\pm$ 0.3)	3654 $\pm$ 307 (1.8 $\pm$ 0.4)	4147 $\pm$ 390 (1.6 $\pm$ 0.3)

Values are mean $\pm$ s.e.m. for 7 rats.

* $P < 0.001$ as compared to other days of the cycle of the same organ (one-way ANOVA).

PBzS density was measured over short time intervals (8 h) and compared to serum oestradiol and progesterone concentrations during days of dioestrus and pro-oestrus (Fig. 1). An increase in PBzS density in both ovary and uterus was associated with an increase in serum oestradiol and progesterone concentrations. The maximal increase in PBzS density in the ovary preceded by 8 h the maximal increase in PBzS density in the uterus.

Ovaries and uteri were grouped according to serum oestradiol and progesterone concentrations without reference to day of the oestrous cycle. Table 2 shows that the increase in PBzS density in the ovary and uterus was positively correlated with the increase in serum oestradiol concentration. A small increase in serum oestradiol concentration was sufficient for a significant increase in PBzS density in the ovary, whereas a significant increase in PBzS density in the uterus was noted only at larger increments of serum oestradiol concentration (Table 2). Such a correlation between PBzS density and serum progesterone concentration could not be drawn (data not shown).

Discussion

The present study demonstrates that PBzS density in ovary, oviduct and uterus alters during the oestrous cycle. We have previously demonstrated that PBzS density in the ovary, oviduct and uterus in immature rats decreases after hypophysectomy and increases after treatment with diethylstilboestrol or PMSG in intact or hypophysectomized rats (Fares *et al.*, 1987). The time-course

Fig. 1. The maximal numbers of binding sites (B_{max}) in the rat ovary and uterus and serum oestradiol and progesterone concentrations throughout dioestrus and pro-oestrus of the 4-day oestrous cycle. Each value represents the mean $\pm$ s.e.m. for 4–5 rats. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared to the value at 09:00 h on the day of dioestrus (Student's t test).

Table 2. Maximal number of binding sites (B_{max}) of PBzS in rat ovary and uterus in relation to serum oestradiol concentration

Oestradiol (pg/ml)	B_{max} (fmol/mg protein)*	
	Ovary	Uterus
≤ 35	1875 ± 320	1810 ± 114
36–50	$3974 \pm 344^\dagger$	2125 ± 375
≥ 51	$3386 \pm 226^\dagger$	$2829 \pm 218^\dagger$

Statistical significance was evaluated by Student's t test.
* B_{max} values of each organ were pooled and subdivided according to the serum oestradiol concentration.
 $^\dagger P < 0.001$ as compared to the B_{max} value observed in rats with serum oestradiol concentration lower than 35 pg/ml.

experiments indicate that the increase in PBzS density is associated with an increase in serum oestradiol and progesterone concentration (Fig. 1). Furthermore, when organs of all rats were pooled without reference to the day of the oestrous cycle, PBzS density in the ovary and uterus was positively correlated with the increasing concentration of serum oestradiol (Table 2). Taken together, these findings suggest that the elevation in serum oestradiol induces the increases in PBzS density in the ovary, oviduct and uterus. It is possible that the slight increase in serum oestradiol concentration starting the night before the day of dioestrus (Smith *et al.*, 1975) initiates the development of PBzS in the ovary and later on in the uterus. The decrease in PBzS density in the ovary, oviduct and uterus on the day of oestrus could result from the significant decrease in concentration of serum oestradiol. The significant increase in secretion of luteinizing hormone beginning on the afternoon of the day of pro-oestrus (Ayalon *et al.*, 1972) could also account for the decrease in PBzS density.

In immature rats PBzS density measured between Days 23 and 27 after birth increased 1.3- and 1.9-fold in the oviduct and uterus, respectively, but remained unaltered in the ovary. This increase in PBzS density may be caused by the high serum oestradiol concentrations measured between Days 9 and 21 after birth (Döhler & Wuttke, 1975). Subcutaneous insertion of a capsule containing diethylstilboestrol on Day 23 after birth for 4 days also caused a significant increase in PBzS density ($P < 0.01$) in the ovary, oviduct and uterus (Fares *et al.*, 1987). Similar treatment with progesterone did not alter the normal increase in PBzS density in the genital organs between Days 23 and 27 after birth (unpublished data), which suggests that progesterone is not involved in the increase in PBzS density. However, in adult rats the increase in PBzS density in the ovary and uterus was also associated with an increase in endogenous serum progesterone concentration. Nevertheless, when organs of all rats were pooled without reference to the day of the oestrous cycle, PBzS density in the ovary and uterus was not correlated with the increase in serum progesterone concentration. This could result from the pulsatile changes in serum progesterone concentration throughout the oestrous cycle (Van der Schoot & de Greef, 1976; Kaneko *et al.*, 1986). Also, Fig. 1 shows fluctuations in serum progesterone concentrations during days of dioestrus and pro-oestrus. Thus, the possibility that progesterone is involved in the development of PBzS in the ovary and uterus cannot be excluded.

The decrease in PBzS density in the rat ovary, oviduct and uterus on the day of oestrus cannot be attributed to atretogenic activity in the ovary, since atretic follicles accumulate on the days of dioestrus and pro-oestrus, with a significant decline on the day of oestrus (Mandl & Zuckerman, 1950; Osman, 1985), or in the uterus, since gap-junction formation is maximal (Garfield *et al.*, 1978) and prostaglandin synthesis is maximal (Brown & Poyser, 1985) on the day of oestrus. The decrease in PBzS density on the day of oestrus is associated with the formation of corpora lutea in the ovary, suggesting that PBzS density in luteal cell membranes is lower compared to granulosa cell membranes.

Several studies have demonstrated that PBzS-selective ligands affect endocrine activity. Treatment with diazepam selectively increases plasma testosterone values in man (Arguelles & Rosner, 1975). Ro 5-4864 increases basal and human chorionic gonadotrophin-stimulated testosterone secretion of rat testis *in vitro* (Wilkinson *et al.*, 1980; Ritta & Calandra, 1986). Diazepam inhibits the secretion of rat prolactin *in vivo* and *in vitro* (Schettini *et al.*, 1984) as well as the secretion of rat thyroid-stimulating hormone *in vitro* (Tapia-Arancibia *et al.*, 1985). Acute administration of low doses of benzodiazepines decreases basal concentrations of plasma corticosterone (Bruni *et al.*, 1980), whereas high doses cause the opposite effect (Marc & Morselli, 1969). These effects of benzodiazepine-selective ligands are associated with high PBzS density in the target endocrine organ, such as testis, hypophysis and adrenal gland (De Souza *et al.*, 1985). Therefore, the presence of PBzS in the ovary, oviduct and uterus suggests a modulatory effect of benzodiazepines on these organs. In a preliminary study we have demonstrated that peripheral benzodiazepine-selective ligands modulate the endocrine activity of various organs in the genital axis. In female rats, diazepam and Ro 5-4864 decrease the oxytocin-dependent contractions of uterine strips (Y. Katz,

F. Fares & M. Gavish, unpublished). In humans, diazepam and Ro 5-4864 increase the secretion of progesterone and oestradiol in placental explant culture (E. R. Barnea, F. Fares & M. Gavish, unpublished).

The mode of benzodiazepinergic action on various organs is as yet unclear. It has been suggested that PBzS are located on the mitochondrial outer membrane (Anholt *et al.*, 1986a, b). It appears that PBzS correspond to the voltage-dependent anion channel, which admits molecules with molecular masses of < 1000 (De Pinto *et al.*, 1985). Porphyrins have been suggested to be the endogenous benzodiazepine ligand for mitochondrial PBzS, since porphyrins inhibit [³H]PK 11195 specific binding at nanomolar range (Verma *et al.*, 1986). Porphyrins are crucial for the activity of cytochrome enzymes, which are involved in the energy-consuming process (Harper *et al.*, 1979) and steroidogenesis (Waterman & Simpson, 1985). This evidence may shed some light on a possible mechanism by which benzodiazepines affect steroidogenesis and cell proliferation in response to hormones.

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