Ovarian contents of immunoreactive β -endorphin and α -N-acetylated opioid peptides in rats*

E. S. Lovegren†, S. J. Zimniski‡ and D. Puett

Reproductive Sciences & Endocrinology Laboratories and Departments of Biochemistry & Molecular Biology, Medicine, and Obstetrics & Gynecology, University of Miami School of Medicine, Miami, Florida 33101, USA

Summary. β -Endorphin was measured by radioimmunoassay in homogenates of ovaries from immature Sprague–Dawley rats (21–29 days of age) and found to be present at levels of about 0.6–0.7 ng/ovary. After administration of PMSG there was approximately a 4-fold increase (2–3 ng/ovary) in total ovarian immunoreactive (ir) β -endorphin 48 h after injection. Analysis of follicular fluid from similarly treated rats indicated about the same amount of ovarian ir- β -endorphin (2–3 ng/ovary) as in ovarian homogenates, suggesting that most of the ir- β -endorphin is localized in follicular fluid of PMSG-primed immature rats. Immature rats were made pseudopregnant by administration of hCG 48 h after PMSG, and at 24 h after injection of hCG there was a slight, but significant and reproducible, increase in the ovarian content of ir- β -endorphin. The serum concentration of ir- β -endorphin was in the range of 1–3 ng/ml and was unaffected by PMSG and PMSG/hCG; likewise, the pituitary content of ir- β -endorphin did not change following administration of gonadotrophins to immature rats. In mature cyclic animals, levels of 2–4 ng ir- β -endorphin/ovary were found, comparable to those in the ovaries of PMSG-primed immature rats, and there were only small changes during the oestrous cycle. In addition to ir- β -endorphin, we also obtained evidence for the presence of α -N-acetylated opioid peptides (endorphins or enkephalins) in the ovaries of PMSG-primed immature and mature rats. The physiological role of the opioid peptides in reproductive tissue is unknown, but they are presumably acting in an autocrine or paracrine fashion.

Keywords: β -endorphin; ovarian opioids; follicular fluid; rat

Introduction

Pro-opiomelanocortin is the precursor to several peptide hormones including β -endorphin, adrenocorticotrophic hormone (ACTH) and the melanocyte-stimulating hormones (Eipper & Mains, 1980; Smith & Funder, 1988). In rat, human and other species, the gene encoding pro-opiomelanocortin is present as a single copy (Eberwine & Roberts, 1983). Although the pituitary contains the highest concentration of pro-opiomelanocortin mRNA (DeBold *et al.*, 1988a), significant amounts have also been detected in ovaries from rats, mice, hamsters, non-human primates and humans (Melner *et al.*, 1986; Chen *et al.*, 1986; Jin *et al.*, 1988). The content of ovarian pro-opiomelano-

*Reprint requests to Dr D. Puett, REPCEND Labs (D-5), University of Miami School of Medicine, P.O. Box 016960, Miami, FL 33101, USA.

†Present address: Department of Medicine, University of Colorado, Denver, CO 80220, USA.

‡Present address: Women's Research Institute, Department of Obstetrics and Gynecology, University of Kansas School of Medicine, Wichita, KS 67214, USA.

cortin mRNA increased about 6-fold 48 h after administration of pregnant mares' serum gonadotrophin (PMSG) to immature rats (Melner *et al.*, 1986; Jin *et al.*, 1988), but after 72 h there was only a marginal elevation (Chen *et al.*, 1986). In cultured granulosa cells from PMSG-primed immature rats, androgens and luteinizing hormone (LH) increased pro-opiomelanocortin mRNA (Melner *et al.*, 1986). Ovarian concentrations also increased during pregnancy in the rat (Chen *et al.*, 1986; Jin *et al.*, 1988), but no changes were found in the different stages of the oestrous cycle (Jin *et al.*, 1988).

Complementing the demonstration of ovarian pro-opiomelanocortin mRNA, immunoreactive (ir) β -endorphin and β -endorphin-like peptides (ir-BEP) have been reported to be present in ovaries from sheep (Lim *et al.*, 1983), mice (Shaha *et al.*, 1984), rats (Tsong *et al.*, 1982; Lolait *et al.*, 1985; Autelitano *et al.*, 1986; Lovegren *et al.*, 1986) and humans (Petraglia *et al.*, 1985; Aleem *et al.*, 1986, 1987; DeBold *et al.*, 1988b). The physiological role of ovarian β -endorphin is unknown, and there are conflicting reports on the amount and relative changes of ir-BEP throughout the oestrous cycle. For example, Shaha *et al.* (1984) found ir-BEP to be fairly constant throughout the mouse oestrous cycle, while Lolait *et al.* (1985) reported significant variations of ir-BEP in the rat oestrous cycle, with the highest concentration occurring on the day of pro-oestrus. In the rat the ovarian concentration of ir-BEP was reported to be lower during pregnancy than in normal cyclic animals (Autelitano *et al.*, 1986), while an opposite conclusion was reached for the mouse (Shaha *et al.*, 1984). Immunocytochemical studies of rat and mouse ovarian sections showed that corpora lutea exhibited the greatest staining and that luteal cells, granulosa cells from large follicles and certain cells of the interstitium also displayed some staining (Shaha *et al.*, 1984; Lolait *et al.*, 1985; Autelitano *et al.*, 1986). In sheep, β -endorphin immunofluorescence-positive cells were noted in follicular cells from oestrous animals, and the culture medium of these cells exhibited ir-BEP; yet, corpora lutea yielded little ir-BEP (Lim *et al.*, 1983). Whether these apparent discrepancies reflect true species differences or result from different methodologies remain to be clarified. In addition to the ovary, pro-opiomelanocortin mRNA and ir-BEP have been identified in the testis and the reproductive tracts of male and female animals (Bardin *et al.*, 1987).

There is also evidence that plasma concentrations of ir-BEP change throughout the menstrual cycle in normal, healthy women, peaking during the periovulatory phase (Vrbicky *et al.*, 1982; Petraglia *et al.*, 1986; Comitini *et al.*, 1989), and it has been suggested that the ovaries contribute to the periovulatory rise (Comitini *et al.*, 1989). Plasma concentrations of ir-BEP are higher in cyclic women than in prepubertal girls or post-menopausal women (Genazzani *et al.*, 1981, 1983).

The purpose of the present investigation was to measure the amounts of ir-BEP in serum, ovary and pituitary of gonadotrophin-primed immature rats and in cyclic adult rats. Preliminary studies were also conducted on the presence of immunoreactive α -N-acetylated opioid peptides (ir-Ac-opioid peptides).

Materials and Methods

Supplies. Na^{125}I (sp. act. 13–17 mCi/ μg) was purchased from Amersham Corp. (Arlington Heights, IL, USA). Gentamycin and Medium 199 were from Grand Island Biological Co. (Grand Island, NY, USA), fetal calf serum from M.A. Bioproducts (Walkersville, MD, USA) and lactoperoxidase (100 IU/mg) from Calbiochem-Behring (San Diego, CA, USA). β -Endorphin (pig sequence), α -N-acetylated β -endorphin (AcBEP, sheep sequence) and the carboxy terminal-specific β -endorphin antiserum were kindly provided by Dr Nicholas Ling (Ling, 1977). The AcBEP antiserum was purchased from Peninsula Laboratories, Inc. (Belmont, CA, USA), and the goat anti-rabbit antiserum used as the second antibody was from Miles Laboratories, Inc. (Naperville, IL, USA). PMSG and bovine serum albumin, Fraction IV (BSA) were obtained from Sigma Chemical Co. (St Louis, MO, USA), and hCG was kindly provided by Dr Robert Canfield (Columbia University, NY, USA). Sprague-Dawley female rats were obtained from Charles River, Inc. (Wilmington, MA, USA).

Iodination and tritium labelling. Lactoperoxidase iodination of β -endorphin and AcBEP was based on a modification of a procedure described by Miyachi *et al.* (1972). Briefly, each peptide was first incubated for 5 min at room temperature with 2 μ g lactoperoxidase, 1 mCi Na^{125}I and 10 μ l 0.003% H_2O_2 in 0.1 M-2-(*N*-morpholino)ethanesulphonic acid, pH 6.0. An additional 10 μ l 0.003% H_2O_2 were added twice and the reaction allowed to continue for 5 min each time. After a total reaction time of 15 min, the mixture was applied to a 0.8×20 cm column of Sephadex G-25 which was developed with 1% acetic acid. To prevent non-specific adsorption of the labelled peptide, the column was precoated with 2 mg BSA and 1 mg poly-L-lysine immediately before use, and the column eluent was collected in polystyrene tubes containing 1 drop of 1% BSA. β -Endorphin was tritium-labelled using reductive methylation as described by Hammonds *et al.* (1981).

Radioimmunoassays. The radioimmunoassay (RIA) for β -endorphin used an antiserum described by Guillemín *et al.* (1977) and was conducted as follows. Samples, 2 or 3 of different volumes with each assayed in duplicate or triplicate, were incubated overnight (16 h) in a volume of 0.4 ml at 4°C with 10^4 c.p.m. ^{125}I -labelled β -endorphin in phosphate-buffered saline (PBS), pH 7.4, containing 0.01% Triton X-100, 0.1% EDTA, β -endorphin antiserum and 1% normal rabbit serum. The second antibody was added in a 0.2 ml volume, followed by an incubation at room temperature for 10 min. Then 0.5 ml 6% polyethylene glycol in PBS was added, and the samples were centrifuged (4°C). Under these conditions, with pig β -endorphin as standard, the sensitivity was about 25 pg, and Student's *t* test established parallelism between standard β -endorphin and serum and tissue homogenates of control and PMSG-primed immature rats. AcBEP and β -endorphin 1–27 cross-react 100% with this antibody and exhibit parallelism to standard β -endorphin. The RIA for AcBEP, with the sheep sequence of AcBEP as standard, was quite similar to that for β -endorphin, the major difference being that the incubation with second antibody was at 4°C for 30 min. The sensitivity was also about 25 pg, and β -endorphin was found to cross-react 1.4% with the AcBEP antibody. The specifications of this antibody report 100% cross-reactivity with the acetylated forms of α -endorphin, γ -endorphin and β -endorphin 1–27 and 31–33% with the acetylated Met- and Leu-enkephalins. Again, parallelism was noted (Student's *t* test) between the AcBEP standard and serum and tissue homogenates. Typically, the intra-assay and inter-assay coefficients of variation were about 5 and 7%, respectively. The RIA results are expressed as mean $\pm$ standard deviation, and N refers to the number of animals used. Student's *t* test and analysis of variance, when appropriate, were used to determine significance ($P < 0.05$).

Animals and tissue preparation. Most studies were performed with gonadotrophin-primed immature Sprague-Dawley female rats (21–29 days old). Animals were injected subcutaneously in the neck with PMSG (20 or 50 i.u.), and then various protocols were followed. To obtain follicular fluid, 27–29-day-old animals were given 20 i.u. PMSG, killed after 48–50 h and the fluid prepared as described below. To study the ovarian content of ir-BEP after administration of 20 i.u. PMSG, 22-day-old rats were killed every 12 h for 60 h after injection. To investigate pseudopregnant animals, 21-day-old rats were acclimatized for 6 days and then injected with PMSG (20 or 50 i.u.); after 48–50 h the animals were injected with saline (controls) or hCG (10 or 25 i.u.) and then killed at 10 and 24 h (the animals are expected to be pseudopregnant only at the latter timepoint). Mature, cyclic animals (230–260 g, 55–60 days of age) were monitored by daily vaginal smears for 2 weeks, pooled according to their day of the cycle, and killed. Ovaries from immature and adult animals were removed and homogenized with a Polytron PT-10 in cold PBS (100 mg tissue/ml PBS). The homogenates were centrifuged for 30 min (4°C) at 48 000 g, and the supernatants were assayed for ir-BEP or ir-AcBEP. Blood was collected to obtain serum, and occasionally other tissues (e.g. pituitary, containing intermediate lobe, as a positive control, liver and spleen) were also processed from immature control animals.

Follicular fluid preparation and granulosa cell culture. Ovaries were removed from immature rats that had received 20 i.u. PMSG 48–50 h earlier, and granulosa cells were prepared using the method described by Campbell (1979), with minor modifications (Koos & Clark, 1982). Following the sequential incubations, the granulosa cells and follicular fluid were expressed into Hepes-buffered Medium 199, containing 1.8 mM-EGTA, by puncturing and exerting gentle pressure on the ovaries. The resulting solution was centrifuged at 100 g for 5 min to pellet the granulosa cells, and the supernatant, which contained the follicular fluid, was frozen at -70°C for subsequent purification and assays. The cells were resuspended in bicarbonate-buffered Medium 199, containing 50 μ g gentamycin/ml, and cultured for 1–96 h in a 5% CO_2 atmosphere. In some experiments hormones (ovine LH and androstenedione), proteins (BSA and fibronectin) and fetal calf serum (1, 5 and 10%) were added. Granulosa cell extracts were prepared by homogenizing the cells in PBS containing several protease inhibitors (e.g. 2 mM-EDTA, 1 mM-phenylmethylsulphonylfluoride and 10 mM-*N*-ethylmaleimide); the homogenates were centrifuged to remove cellular debris. The follicular fluid-containing medium was assayed directly or partly purified using C-18 SepPak cartridges (Waters Associates, Inc., Milford, MA, USA) based on a modification of the procedure described by Cahill *et al.* (1983). The endorphin-like peptides were eluted with 90% ethanol containing 4% acetic acid, dried and applied to a SynChrom reverse-phase column with 15% CH_3CN , 0.1% TFA. The ir-BEP fractions were eluted by a gradient to 80% CH_3CN , 0.1% TFA (Lovegren *et al.*, 1986). Fractions were dried and assayed with the β -endorphin antiserum.

To test for the stability of β -endorphin in granulosa cell cultures, the peptide was added to a final concentration of 10 ng/ml to wells containing either 10^6 cells/well in Medium 199 or just Medium 199. Following incubation at 37°C for 1, 19 and 24 h, media were collected and the amount of immunoreactive β -endorphin determined by RIA. Two wells were used for each sample, and the medium from each well was assayed in triplicate. The results are given as percentage of recovered immunoreactive β -endorphin.

Results

Follicular fluid from PMSG-stimulated immature rats

Acidic conditions, which are widely used in the preparation of ovarian opioid peptides (Lim *et al.*, 1983; Shaha *et al.*, 1984; Autelitano *et al.*, 1986), were found to be quite detrimental to the amount of type of immunoreactive peptide derived from follicular fluid. For example, several independent preparations of follicular fluid, expressed into Medium 199 and then assayed, yielded ir-BEP concentrations between 2 and 3 ng/ovary, and even higher levels (4–7 ng/ovary) were found of ir-Ac-opioid peptides (Table 1). In contrast, quite different results were obtained from follicular fluid exposed to acidic conditions before assay. In one experiment follicular fluid-containing medium was acidified to pH 2.5 with HCl, incubated for several minutes on ice, and then the pH was raised to 7.4. Assays on this sample and on a control maintained at neutral pH for the same time showed a 60% loss of ir-BEP following this brief exposure to acidic conditions. In another experiment in which follicular fluid was partly purified using C-18 SepPak cartridges and reverse-phase HPLC under acidic conditions, the majority of the immunoreactive material co-eluted with standard β -endorphin; only a minor component co-eluted with standard AcBEP (Lovegren *et al.*, 1986). In a separate experiment follicular fluid was chromatographed on a Sephadex G-25 column that was developed with 5% acetic acid, and no ir-Ac-opioid peptides could be detected. We therefore conclude that follicular fluid from PMSG-stimulated immature rats contains immunoreactive opioid and α -N-acetylated opioid peptides and that acidic conditions greatly diminish the amount of these peptides.

Table 1. Concentrations of ir-BEP and ir-Ac-opioid peptides in follicular fluid of PMSG-stimulated immature rats*

Experiment no.	No. of rats	ir-BEP (ng/ovary)	ir-Ac-opioid peptides (ng/ovary)
1	20	1.9 $\pm$ 0.1	4.0 $\pm$ 0.7
2	17	3.2 $\pm$ 0.1	7.3 $\pm$ 1.4
3	16	3.0 $\pm$ 0.3	7.3 $\pm$ 0.4
4	25	2.1 $\pm$ 0.1	5.2 $\pm$ 1.0
5	25	2.1 $\pm$ 0.1	4.6 $\pm$ 0.4

*Rats (27–29 days old) were treated with 20 i.u. PMSG and killed 48 h later. Follicular fluid was expressed into Hepes-buffered Medium 199, containing 1.8 mM-EGTA, and triplicate determinations were made on each of 3 doses (data given as mean $\pm$ s.d.).

Ovaries of gonadotrophin-treated immature rats

In preliminary studies, it was found that homogenization of rat ovaries in cold (4°C) 10% (v/v) acetic acid, i.e. conditions similar to those used by others in the preparation of rat brain homogenates (Ogawa *et al.*, 1979), led to a considerable loss (up to 80%) of ir-BEP, relative to that obtained following rapid homogenization in cold PBS. If, however, the ovaries were homogenized in cold 10% (v/v) acetic acid containing a mixture of protease inhibitors (20 mM-EDTA, 20 mM-N-ethylmaleimide, 1 mM-phenylsulphonylfluoride and 0.5 mM-dithiothreitol), there was only a 10% loss in the content of ir-BEP relative to homogenization in cold PBS. The above mixture of protease inhibitors had no effect on the measured amount of ir-BEP obtained with homogenization in cold PBS. The addition of [3 H] β -endorphin to ovarian homogenates in cold PBS at pH 7.4

yielded a 95% recovery through the centrifugation step. Based upon these results, ovaries were handled rapidly and routinely homogenized in cold PBS at pH 7.4. In two separate experiments the ovarian content of ir-BEP in immature rats was 0.6 ± 0.1 and 0.7 ± 0.1 ng/ovary. As expected, the pituitary of immature rats (29 days old) had the highest level of ir-BEP (546 ± 45 ng/mg protein, $N = 24$), and the amount of ir-BEP (expressed as ng/mg protein) in ovaries was about 4 times that in spleen and liver; a typical serum concentration was 2.2 ± 0.2 ng/ml ($N = 24$).

The ovarian content of ir-BEP at various times after an injection of 20 i.u. PMSG is shown in Fig. 1. Expressed per ovary, there was a significant increase in ir-BEP after administration of PMSG. However, if the data are given as amount of ir-BEP per mg protein (determined on the same solution as used for RIA) or per unit tissue wet weight, there was a decrease from 0 to 12 h (a 40% decrease based on mg ovarian protein and a 27% decrease based on wet ovarian weight), and from 12 to 60 h the values were fairly constant. At 48 h after PMSG injection, there was no apparent dose-response of ir-BEP to PMSG between 20 and 50 i.u.: the fold-increase in ir-BEP content per ovary was 3.7 ± 0.9 with 20 i.u. PMSG and 3.8 ± 0.6 with 50 i.u. PMSG (not significantly different). This result is not surprising in view of the pharmacological doses of PMSG that were used. The content of ir-BEP in ovarian homogenates of PMSG-primed immature rats was almost identical with that in follicular fluid of similarly treated animals.

Fig. 1. Ovarian content of ir-BEP of immature rats (22 days old) receiving PMSG. At time zero the animals were injected with 20 i.u. PMSG and groups ($N = 4$) were killed every 12 h up to 60 h. All values significantly differed from control (0 h): ^a $P < 0.02$, ^b $P < 0.01$, ^c $P < 0.001$.

The amounts of ir-BEP and ir-Ac-opioid peptides were measured in serum, ovaries and pituitary of immature rats 48 h after injection of 50 i.u. PMSG (Table 2). Expressed per ovary, both ir-BEP and ir-Ac-opioid peptides increased 4.4-fold and 2.5-fold, respectively, following PMSG administration. However, when determined per mg ovarian protein or per wet ovarian weight, there was a reduction comparable to that obtained with 20 i.u. PMSG. In contrast, serum and pituitary concentrations were unaffected by injection of PMSG.

As shown in Fig. 2, there was an increase in the ovarian content of ir-BEP 24 h after injection of hCG, but there was no evidence of a dose-response at these pharmacological doses of gonadotrophins. Serum concentrations ($1.2\text{--}1.5$ ng ir-BEP/ml) and pituitary content ($190\text{--}250$ ng ir-BEP/pituitary) were relatively constant at all time points and hormonal additions.

There were no detectable concentrations of ir-BEP in media and cell extracts from granulosa cells, cultured under a variety of conditions. From the sensitivity of the assay this would indicate that ir-BEP, if present, is < 0.25 pg/ml. (Likewise, ir-ACTH in the medium was low, e.g. < 0.2 ng/ml). To monitor the stability of β -endorphin in the cell cultures, β -endorphin was added to the medium of cultured granulosa cells and to medium alone (i.e. no cells). The results in Table 3 show that the peptide was quite stable in the absence of cells, but $> 40\%$ of the added ir-BEP was lost in just 1 h of culture with granulosa cells and about 90% after 24 h.

Table 2. Concentrations of ir-BEP and ir-Ac-opioid peptides in serum, pituitary and ovaries of immature rats receiving saline or PMSG†

Serum tissue	Treatment	ir-BEP (ng)	ir-Ac-opioid peptides (ng)
Serum	Saline	2.59 ± 0.52	2.77 ± 0.42
	PMSG	1.71 ± 0.34	2.29 ± 0.75
Ovary	Saline	0.59 ± 0.02	0.90 ± 0.02
	PMSG	2.58 ± 0.18*	2.24 ± 0.43*
Pituitary	Saline	223 ± 4	949 ± 49
	PMSG	194 ± 5	1038 ± 44

†Immature rats received a saline injection or 50 i.u. PMSG and were killed 48 h later. Values are mean ± s.d. for 13 control (saline) and 6 PMSG-treated rats and are expressed per ml serum and per organ for pituitary and ovary.

* $P < 0.05$ compared with saline value.

Fig. 2. Effects of PMSG and hCG on ovarian ir-BEP in immature rats. [All animals were injected with PMSG, then 48 h later with saline (□) or hCG (■). After the last injection animals were killed at 10 h and 24 h. One group that received saline was killed at 2 h to serve as a control for the 10- and 24-h time points.] (a) Combined results of 2 experiments (total N = 7 per group) using 20 i.u. PMSG and 10 i.u. hCG. (b) Results of one experiment (N = 6 per group) using 50 i.u. PMSG and 25 i.u. hCG. Values significantly different from the 2-h control are indicated: ^a $P < 0.05$, ^b $P < 0.001$, ^c $P < 0.03$, ^d $P < 0.01$. In addition, at 24 h the ovarian content of ir-BEP was greater in rats receiving hCG compared to the corresponding saline control: $P < 0.005$ (a) and $P < 0.007$ (b).

Mature, cyclic animals

Initial assays indicated that the ovaries of mature animals contained 2–4 ng ir-BEP/ovary, or 72–78 ng/g tissue weight, that changed slightly with the stage of the cycle, the lowest amount occurring during oestrus. Table 4 shows a summary of two assays run in parallel on ir-BEP and ir-Ac-opioid peptides; the amounts of ir-BEP appear to be greater than those of the ir-Ac-opioid peptides.

Discussion

These results demonstrate that the total amount of ir-BEP in the ovary of immature rats increases about 4-fold 48 h after administration of PMSG, and at this time most of the ir-BEP appears to be

Table 3. Degradation of β -endorphin by cultured granulosa cells*

Incubation time (h)	% ir β -endorphin recovered	
	No cells	With cells
1	72.1 $\pm$ 6.0	57.1 $\pm$ 2.8
19	78.7 $\pm$ 6.1	15.2 $\pm$ 4.2
24	86.0 $\pm$ 11.1	9.0 $\pm$ 1.6

* β -Endorphin (10 ng/ml) was added to wells containing Medium 199 with or without granulosa cells and incubated at 37°C for the indicated times. At the end of each incubation the medium was removed and assayed for ir-BEP. Values are mean $\pm$ s.d.

Table 4. Ovarian content of ir-BEP and ir-Ac-opioid peptides in cyclic rats*

Day of cycle	ir-BEP (ng/ovary)†	ir-Ac-opioid peptides (ng/ovary)‡
Metoestrus	2.76 $\pm$ 0.12 ^a	1.79 $\pm$ 0.08 ^a
Dioestrus	2.25 $\pm$ 0.04 ^b	1.41 $\pm$ 0.08
Pro-oestrus	2.78 $\pm$ 0.06 ^a	1.43 $\pm$ 0.30
Oestrus	1.86 $\pm$ 0.10 ^c	1.29 $\pm$ 0.09 ^b

*These data are from 2 assays performed in parallel on homogenates that were thawed twice; the corresponding mean values after the first thaw were 3.8, 3.1, 3.0 and 2.8 ng ir-BEP/ovary for metoestrus, dioestrus, pro-oestrus and oestrus, respectively, indicating a decrease in the content of ir-BEP with freeze-thawing. The 4 animals in each group were killed on the morning of the indicated day of the cycle.

†Values with different letters (a, b, c) are significantly different from each other ($P < 0.004$) by analysis of variance.

‡Only the two values indicated with different letters (a, b) are significantly different from each other ($P < 0.004$) by analysis of variance.

localized in follicular fluid. Thus, under these conditions it appears that BEP does not accumulate intracellularly. The measured increase in the amount of immunoreactive ovarian peptide is consistent with the increases measured in ovarian pro-opiomelanocortin mRNA levels, expressed per μ g total RNA, of comparably treated animals (Melner *et al.*, 1986; Jin *et al.*, 1988). None the less, these results do not demonstrate a specific increase of ir-BEP mediated by PMSG administration to immature rats since, when expressed per unit wet weight of tissue or per mg soluble ovarian protein, the specific contents decrease. A proper reference state for the immature ovary, e.g. expressed per whole ovary, per wet tissue weight, or per mg protein, is difficult to define, since one is comparing a tissue which contains only primordial follicles with that from a PMSG-stimulated immature animal

in which there is a pronounced proliferation of all types of follicles, and particularly antral follicles containing significant quantities of follicular fluid and occasional corpora lutea. Also, Kew *et al.* (1989) have shown that the amount of proenkephalin mRNA in rat ovary is not an accurate indicator of the amount of peptide.

Shaha *et al.* (1984), using a different experimental model, reported that the ovaries of 24-day-old immature mice exhibited only faintly positive immunostainable β -endorphin in the interstitium, none being observed in the follicles. Corpora lutea from mice that received 30 i.u. PMSG at 22 days of age and killed 60 h later showed the most intense staining for ir-BEP; moreover, secondary and large antral follicles stained positively, and the interstitium was more heavily stained than that from control animals. In the normal cyclic adult rat, Lolait *et al.* (1985) also found the most intense immunofluorescence staining of cells in the corpora lutea, but this was not observed in sheep ovary (Lim *et al.*, 1983). Our results with PMSG-primed immature rats are qualitatively consistent with the findings of Shaha *et al.* (1984) on PMSG-primed immature mice. Also, the small increase we found in ir-BEP from pseudopregnant immature animals relative to PMSG-primed immature animals is consistent with immunostainable β -endorphin in rodent corpora lutea (Shaha *et al.*, 1984; Lolait *et al.*, 1985). Since we are dealing with total ovarian homogenates, increased synthesis in localized ovarian compartments, such as corpora lutea, may be difficult to quantitate.

Much of the ovarian ir-BEP in PMSG-treated immature rats appears to be localized in the follicular fluid, and we also found evidence for the presence of α -N-acetylated opioid peptides in ovaries, as well as in serum. The inability to detect ir-BEP in cultured granulosa cells from PMSG-primed immature rats can probably be attributed to the rapid degradation of the peptide, perhaps by secreted proteases or lysed cells. It has also been suggested that proenkephalin products have a relatively high turnover rate in the ovaries of intact rats (Kew *et al.*, 1989).

In mature cyclic rats we found only small, if any, differences in the amount of ir-BEP or ir- α -N-acetylated peptides at the various stages of the cycle. This finding is in agreement with reports on pro-opiomelanocortin mRNA levels in cyclic mice (Shaha *et al.*, 1984), but is in marked contrast to the report by Lolait *et al.* (1985) who found maximal ir-BEP ovarian levels at pro-oestrus, minimal values at oestrus and metoestrus, and intermediate levels at dioestrus in the rat. Our values for ir-BEP ranged between 2 and 4 ng/ovary, comparable to that of the PMSG-primed immature rat and considerably higher than the range of 0.1–0.4 ng/ovary reported by Lolait *et al.* (1985). The amounts of rat ovarian ir-BEP found by Lolait *et al.* (1985) are some 2–8 times higher than those reported by Shaha *et al.* (1984) for mouse ovaries, and so the ovarian ir-BEP contents found in the present study represent the largest values reported for rodent ovary. Others have homogenized animal ovaries in the presence of 0.1 M-HCl (Lim *et al.*, 1983; Autelitano *et al.*, 1986), a mixture of 0.1 M-HCl, 1.2 M-formic acid, and 0.13 M-trifluoroacetic acid (Shaha *et al.*, 1984), or have used 0.1 M-HCl and a boiling step (Lolait *et al.*, 1985) to prepare fractions for radioimmunoassay. Our results showed that homogenization in the presence of 10% acetic acid yielded a much lower ir-BEP content than homogenization in cold PBS or in 10% acetic containing a mixture of protease inhibitors. However, homogenization of rat ovaries in the presence of a combination of acetic acid (10%) and acetone (80%) gave a somewhat greater ir-BEP content than with PBS alone (unpublished data). Whether the apparent discrepancies reported in the various studies reflect true species differences or arise from the different extraction conditions used or antibody specificity is not known at this time.

In conclusion, we have quantified the amount of ir-BEP in ovaries from control, PMSG-primed, and pseudopregnant immature rats and from adult rats. Evidence is also given for the presence of α -N-acetylated ir-endorphins or enkephalins in rat ovary. No specific information can be provided on the significance of the presence of α -N-acetylated peptides in rat ovary; whether this post-translational modification serves to abolish opiate-like receptor binding or to promote binding to non-opioid receptors (Shahabi *et al.*, 1990) remains to be determined. The function of ovarian opioid peptides is not known, but they presumably act in an autocrine or paracrine role in ovary and testis (see Bardin *et al.*, 1987). It has been suggested that the ovaries may contribute to

the plasma ir-BEP levels during the periovulatory stage of the human menstrual cycle (Comitini *et al.*, 1989).

We thank Dr Nicholas Ling for providing peptide standards and the β -endorphin antiserum; and Dr Robert D. Koos for helpful guidance on the preparation of granulosa cells and follicular fluid. This research was supported by the National Institutes of Health (DK33973).

References

- Aleem, F.A., Omar, R.A. & Eltabbakh, G.H. (1986) Immunoreactive β -endorphin in human ovaries. *Fert. Steril.* **45**, 507–511.
- Aleem, F.A., Eltabbakh, G.H., Omar, R.A. & Southren, A.L. (1987) Ovarian follicular fluid β -endorphin levels in normal and polycystic ovaries. *Am. J. Obstet. Gynecol.* **156**, 1197–1200.
- Autelitano, D.J., Lolait, S.J., Smith, A.I. & Funder, J.W. (1986) Pregnancy-associated changes in ovarian immunoreactive β -endorphin in rats. *J. Endocr.* **108**, 343–350.
- Bardin C.W., Chen, C.-L.C., Morris, P.L., Gerendai, I., Boitani, C., Liotta, A.S., Margioris, A. & Krieger, D.T. (1987) Proopiomelanocortin-derived peptides in testis, ovary, and tissues of reproduction. *Recent Prog. Horm. Res.* **43**, 1–25.
- Cahill, C., Matthews, J. & Akil, H. (1983) Human plasma β -endorphin-like peptides: a rapid, high recovery extraction technique and validation of radioimmunoassay. *J. clin. Endocr. Metab.* **56**, 992–997.
- Campbell, K.L. (1979) Ovarian granulosa cells isolated with EGTA and hypertonic sucrose: cellular integrity and function. *Biol. Reprod.* **21**, 773–786.
- Chen, C.-L.C., Chang, C.-C., Krieger, D.T. & Bardin, C.W. (1986) Expression and regulation of proopiomelanocortin-like gene in the ovary and placenta: comparison with the testis. *Endocrinology* **118**, 2382–2389.
- Comitini, G., Petraglia, F., Facchinetti, F., Monaco, M., Volpe, A. & Genazzani, A.R. (1989) Effect of oral contraceptives or dexamethasone on plasma β -endorphin during the menstrual cycle. *Fert. Steril.* **51**, 46–50.
- DeBold, C.R., Nicholson, W.E. & Orth, D.N. (1988a) Immunoreactive proopiomelanocortin (POMC) peptides and POMC-like messenger ribonucleic acid are present in many rat nonpituitary tissues. *Endocrinology* **122**, 2648–2657.
- DeBold, C.R., Menefee, J.K., Nicholson, W.E. & Orth, D.N. (1988b) Proopiomelanocortin gene is expressed in many normal human tissues and in tumors not associated with ectopic adrenocorticotropin syndrome. *Molec. Endocrinol.* **2**, 862–870.
- Eberwine, J.H. & Roberts, J.L. (1983) Analysis of POMC gene structure and function. *DNA* **2**, 1–8.
- Eipper, B.A. & Mains, R.E. (1980) Structure and biosynthesis of proadrenocorticotropin/endorphin and related peptides. *Endocr. Rev.* **1**, 1–27.
- Genazzani, A.R., Facchinetti, F., Ricci-Danero, G., Parrini, D., Petraglia, F., La Rosa, R. & D'Antona, N. (1981) Beta-lipotropin and beta endorphin in physiological and surgical menopause. *J. endocrinol. Invest.* **4**, 375–378.
- Genazzani, A.R., Facchinetti, F., Pintor, C., Parrini, D., Petraglia, F., Bagnoli, F. & Corda R. (1983) Proopiomelanocortin-related peptide plasma levels throughout prepuberty and puberty. *J. clin. Endocr. Metab.* **57**, 56–61.
- Guillemin, R., Ling, N. & Vargo, T. (1977) Radioimmunoassays for α -endorphin and β -endorphin. *Biochem. Biophys. Res. Commun.* **77**, 361–366.
- Hammonds, R.G., Jr, Ling, N. & Puett, D. (1981) Preparation of tritium labeled β -endorphin suitable for studying brain receptor interactions. *Biochem. Biophys. Res. Commun.* **98**, 211–218.
- Jin, D.F., Muffly, K.E., Okulicz, W.C. & Kilpatrick, D.L. (1988) Estrous cycle- and pregnancy-related differences in expression of the proenkephalin and proopiomelanocortin genes in the ovary and uterus. *Endocrinology* **122**, 1466–1471.
- Kew, D., Jin, D.F., Kim, F., Laddis, T. & Kilpatrick, D.L. (1989) Translation status of proenkephalin mRNA in the rat reproductive system. *Molec. Endocrinol.* **3**, 1191–1196.
- Koos, R.D. & Clark, M.R. (1982) Production of 6-keto prostaglandin $F_{1\alpha}$ by rat granulosa cells *in vitro*. *Endocrinology* **111**, 1513–1518.
- Lim, A.T., Lolait, S., Barlow, J.W., Zois, I., Toh, B.H. & Funder, J.W. (1983) Immunoreactive β -endorphin in sheep ovary. *Nature, Lond.* **303**, 709–711.
- Ling, N. (1977) Solid phase synthesis of porcine α -endorphin and γ -endorphin, two hypothalamic-pituitary peptides with opiate activity. *Biochem. Biophys. Res. Commun.* **74**, 248–255.
- Lolait, S.J., Autelitano, D.J., Lim, A.T.W., Smith, A.I., Toh, B.H. & Funder, J.W. (1985) Ovarian immunoreactive β -endorphin and estrous cycle in the rat. *Endocrinology* **117**, 161–168.
- Lovegren, E., Zimniski, S. & Puett, D. (1986) Immunoreactive β -endorphin in the gonadotropin-primed immature rat ovary. *Advances in Gene Technology: Molecular Biology of the Endocrine System, ICSU Short Reports* **4**, 48–49.
- Melner, M.H., Young, S.L., Czerwiec, F.S., Lyn, D., Puett, D., Roberts, J.L. & Koos, R.D. (1986) The regulation of granulosa cell proopiomelanocortin messenger ribonucleic acid by androgens and gonadotropins. *Endocrinology* **119**, 2082–2088.
- Miyachi, Y., Vaitukaitis, J.L., Nieschlag, E. & Lipsett, M.B. (1972) Enzymatic radioiodination of gonadotropins. *J. clin. Endocr. Metab.* **34**, 23–28.
- Ogawa, N., Panerai, A.E., Lee, S., Forsbach, G., Havlicek, V. & Friesen, H.G. (1979) β -Endorphin concentration in the brain of intact and hypophysectomized rats. *Life Sci.* **25**, 317–326.

- Petraglia, F., Segre, A., Facchinetti, F., Campanini, D., Ruspa, M. & Genazzani, A.R.** (1985) β -Endorphin and met-enkephalin in peritoneal and ovarian follicular fluids of fertile and postmenopausal women. *Fert. Steril.* **44**, 615–621.
- Petraglia, F., Di Meo, G., De Leo, V., Nappi, C., Facchinetti, F. & Genazzani, A.R.** (1986) Plasma β -endorphin levels in anovulatory states: Changes after treatment for the induction of ovulation. *Fert. Steril.* **45**, 185–190.
- Shaha, C., Margioris, A., Liotta, A.S., Krieger, D.T. & Bardin, C.W.** (1984) Demonstration of immunoreactive β -endorphin- and γ_3 -melanocyte-stimulating hormone-related peptides in the ovaries of neonatal, cyclic, and pregnant mice. *Endocrinology* **115**, 378–384.
- Shahabi, N.A., Linner, K.M. & Sharp, B.M.** (1990) Murine splenocytes express a naloxone-insensitive binding site for β -endorphin. *Endocrinology* **126**, 1442–1448.
- Smith, A.I. & Funder, J.W.** (1988) Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. *Endocr. Rev.* **9**, 159–179.
- Tsong, S.-D., Phillips, D.M., Halmi, N., Krieger, D. & Bardin, C.W.** (1982) β -endorphin is present in the male reproductive tract of five species. *Biol. Reprod.* **27**, 755–764.
- Vrbicky, K.W., Baumstark, J.S., Wells, I.C., Hilgers, T.W., Kable, W.T. & Elias, C.J.** (1982) Evidence for the involvement of β -endorphin in the human menstrual cycle. *Fert. Steril.* **38**, 701–704.

Received 13 March 1990