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Expression of the enkephalin precursor gene in rat Sertoli cells

Regulation by follicle-stimulating hormone

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Searching for somatic cells expressing the preproenkephalin (A) gene in the testis, we have isolated Sertoli cells from the testes of 20-day-old rats. Cultured Sertoli cells contained a single species (about 1.5 kb) of preproenkephalin mRNA, and follicle-stimulating hormone (FSH) transiently increased the mRNA abundance to a maximum (about 30 molecules per cell) at 12 h. Various compounds that activate the cyclic AMP system in Sertoli cells similarly increased the abundance of preproenkephalin mRNA. Moreover, FSH increased intracellular Met-enkephalin immunoreactive peptides in Sertoli cells. Thus, the preproenkephalin gene expression in Sertoli cells is positively regulated by FSH through the cyclic AMP system.

Preproenkephalin; mRNA; FSH; cyclic AMP; Autocrine; (Rat Sertoli cell)

1. INTRODUCTION

Enkephalins (Met- and Leu-enkephalin) are widely distributed throughout the central and peripheral nervous systems, and modulate various neuronal functions. Recent studies have shown that mRNA encoding preproenkephalin (A) (ppEnk; the precursor of Met- and Leu-enkephalin) is found not only in the neuronal tissues but also in various non-neuronal tissues such as the heart [1], lymphocytes [2], and reproductive systems [3].

In the testis, a large amount of ppEnk mRNA with a unique size of 1.9 kilobases (kb) is present in the germ cells [4,5]. Little is known, however, about ppEnk gene expression in testicular somatic (non-germinal) cells such as Leydig (i.e. testosterone-secreting interstitial cells), Sertoli (i.e. inhibin-secreting intratubular cells) and peritubular myoid cells. We report here that cultured rat Sertoli cells contain ppEnk mRNA whose abundance is positively regulated by follicle-stimulating hormone (FSH).

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2. MATERIALS AND METHODS

2.1. *Materials*

Rat FSH (NIH-rFSH12VO7) and luteinizing hormone (NIH-rLH12VO7) were generously provided by the National Hormone and Pituitary Program, NIDDK, NIH. Forskolin was donated by Nihon Kayaku Co. 8-Bromo-cyclic AMP, norepinephrine and dexamethasone were purchased from Sigma. C6 glioma cells were obtained from the American Type Culture Collection.

2.2. *Preparation of Sertoli cell-enriched cultures*

Sertoli cell-enriched cultures were prepared from 20-day-old Wistar strain rats according to Tung et al. [6]. Sertoli cells were plated into 100 × 20 mm tissue culture dishes (Falcon 3003) at a density of $1–2 \times 10^6$ aggregates. The cells were incubated at 33°C for 4–5 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and non-essential amino acids under a humidified atmosphere of 95% air/5% CO_2, and then treated with hormones or chemicals for various periods of time. The culture system was verified by morphological changes of Sertoli cells in response to FSH and 8-bromo-cyclic AMP [6]. Final cell densities were

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$1\text{-}2 \times 10^7$/dish, and viability of the harvested Sertoli cells was more than 95% by trypan blue exclusion test.

2.3. *RNA extraction and blot hybridization*

Total cellular RNA was extracted from cultured Sertoli cells by the differential ethanol precipitation method [7], and quantified by ultraviolet absorption at 260 nm. Yields of total RNA were 70 ± 0.8 μg/dish. ppEnk and β-actin mRNAs were analyzed by Northern blotting as in [8]: Total RNA was electrophoresed on a 6% formaldehyde/1.2% agarose gel, and blotted onto a nylon membrane (Hybond-N, Amersham). RNA blots were hybridized with ^{32}P-labeled probes of ppEnk cDNA (pRPE2) [8] and β-actin DNA (a gift from Dr B. Paterson, NCI/NIH). Quantitative dot-blot analysis of ppEnk mRNA was performed using the SP6-transcribed ppEnk RNA standard [5,9]. The autoradiogram exposed at −80°C with an intensifying screen was scanned with a densitometer (Shimadzu model CS-9000) for quantitation of the hybridization signals.

2.4. *Met-enkephalin radioimmunoassay*

Sertoli and C6 glioma cells were plated into 60 × 15 mm tissue culture dishes (Falcon 3002). Met-enkephalin (Met-enk) immunoreactivity in the cell extract was determined by radioimmunoassay using ^{125}I-Met-enk (Amersham) and RB4 antiserum (a gift from Dr S. Sabol, NHLBI/NIH) [10,11]. An aliquot of the extract was treated sequentially with trypsin and carboxypeptidase B to release Met-enkephalin from the unprocessed precursor [10,12]. The protein content of cell lysates was determined by bicinchoninic acid method [13] using bovine serum albumin as a standard.

3. RESULTS

Cultured Sertoli cells contained a single species of ppEnk mRNA of 1.5 kb length; treatment with FSH for 6 h at 0.3 and 1.0 μg/ml increased the ppEnk mRNA levels to 12- and 13-times the control, respectively (fig.1A). The size of ppEnk mRNA in Sertoli cells is identical to that present in other ppEnk gene-expressing tissues except for testicular germ cells [4,5]. The abundance of

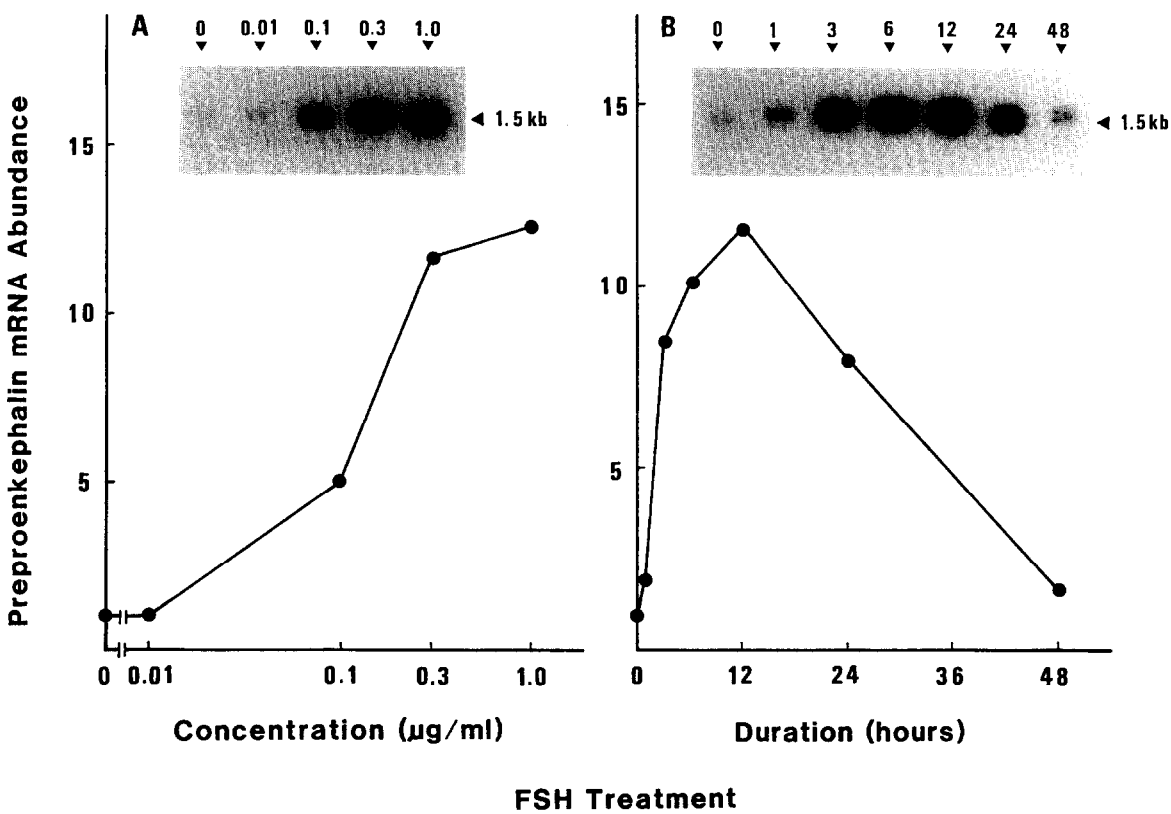

Fig.1. Effect of FSH on ppEnk mRNA abundance in cultured Sertoli cells. (A) Dose-response curve. Cultured Sertoli cells were incubated with various concentrations of FSH for 6 h. (B) Time course. Sertoli cells were treated with FSH (0.5 μg/ml) for various periods prior to harvesting. Autoradiographic densities are given in arbitrary units. Insets; autoradiograms of 6 h exposure.

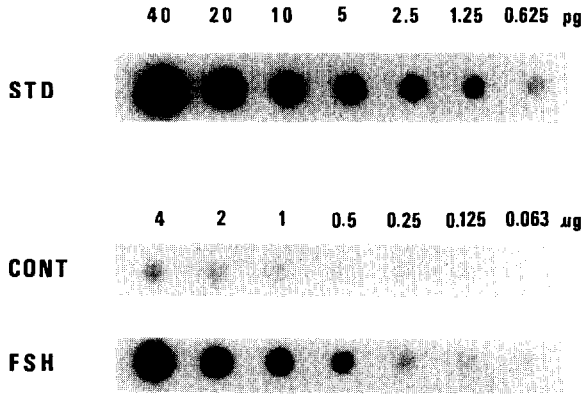

Fig.2. Quantitative dot-blot analysis of ppEnk mRNA in Sertoli cells cultured in the absence or presence of FSH. Sertoli cells were incubated in the absence (CONT) or presence of FSH (FSH) at 0.5 μg/ml for 12 h. Seven known amounts of SP6-transcribed ppEnk RNA (STD) and of total RNA prepared from the Sertoli cells were hybridized with the ^{32}P-labeled ppEnk cDNA probe. 1 pg of the 950-base ppEnk RNA standard corresponds to 3.1×10^{-18} mol. An autoradiogram of 6 h exposure is shown. Calculated values are in the text.

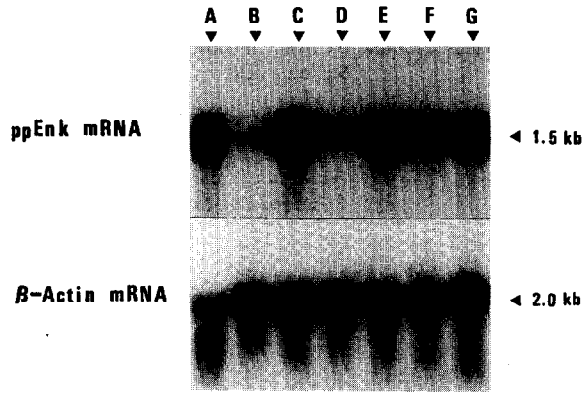

Fig.3. Effects of various compounds that activate cyclic AMP system on ppEnk mRNA abundance in cultured Sertoli cells. Sertoli cells were treated with various compounds for 7 h (B–G). Lanes: A, rat cerebral cortex; B, untreated control; C, FSH (0.5 μg/ml); D, luteinizing hormone (0.5 μg/ml); E, forskolin (10 μM); F, 8-bromo-cyclic AMP (0.5 mM); G, norepinephrine (10 μM). RNA blots were initially hybridized with the ^{32}P-labeled ppEnk cDNA probe, and then re-probed with ^{32}P-labeled β-actin DNA after stripping ppEnk cDNA probe. Autoradiograms of ppEnk mRNA and β-actin mRNA are of 16 and 1 h exposure, respectively. Numbers at right are the sizes of hybridized signals.

ppEnk mRNA in FSH-treated cells showed an apparent peak at 12 h, and declined to near-control level in 48 h (fig.1B). The time course resembles that of the norepinephrine-induced ppEnk mRNA change in C6 glioma cells as reported in [9].

The abundances of ppEnk mRNA in control and FSH-stimulated Sertoli cells were quantified by dot-blot analysis using synthetic ppEnk RNA as a standard (fig.2). The ppEnk mRNA abundances in unstimulated (control) and maximally stimulated cells were 0.36 and 5.7 pg/μg total RNA, respectively, when corrected for the difference in size between natural ppEnk mRNA (1.5 kb) and synthetic ppEnk RNA (0.95 kb). These correspond to 2.0 (control) and 29.5 (FSH-stimulated) ppEnk mRNA molecules per cell.

FSH exerts its effect through the receptor-mediated cyclic AMP system in Sertoli cells. Therefore, effects of various compounds that activate the cyclic AMP system were examined (fig.3, upper panel). FSH, forskolin (an adenylate cyclase activator), 8-bromo-cyclic AMP (a membrane-permeable analogue of cyclic AMP) and norepinephrine (a β-adrenergic agonist) markedly increased the ppEnk mRNA abundance, indicating that FSH exerts its effect through the cyclic AMP system. On the other hand, luteinizing hormone (i.e. the pituitary hormone that regulates the functions of Leydig cells) caused a much smaller increase in the ppEnk mRNA level. These compounds failed to exert appreciable effects on the β-actin mRNA levels used as a control (fig.3, lower panel).

To examine whether FSH-induced ppEnk mRNA is translatable into ppEnk that is further processed to enkephalins and related peptides, we then determined Met-enk immunoreactivity in Sertoli cells. Very little Met-enk immunoreactivity was detected in the control (unstimulated) Sertoli cells, but it was markedly increased by treatment with FSH or forskolin (table 1). The Met-enk immunoreactivity after limited proteolysis was similar to that before the treatment, suggesting that ppEnk is almost completely processed. On the other hand, C6 glioma cells contained some processed Met-enk, and limited proteolysis increased Met-enk immunoreactivity 2.1- and 4.6-fold in unstimulated and stimulated cells, respectively (table 1). These results suggest that Sertoli cells, unlike C6 cells, possess the machinery for processing the newly synthesized ppEnk (e.g. secretory granules containing precursor-processing enzymes).

Table 1

Met-enkephalin immunoreactivity in cultured Sertoli cells and C6 glioma cells

Additions	Protein (μg/dish)	Met-enkephalin immunoreactivity (fmol/mg protein)	
		Before digestion	After digestion
Sertoli cells			
None	568 ± 8	< 8	< 8
FSH	755 ± 41	50 ± 7	58 ± 5
Forskolin	818 ± 15	47 ± 3	45 ± 5
C6 glioma cells			
None	1842 ± 17	14 ± 3	30 ± 4
Forskolin + Dex	1813 ± 21	11 ± 2	51 ± 3

Sertoli cells were cultured for 5 days, and treated with FSH (0.5 μg/ml) or forskolin (10 μM) for 24 h before harvesting. C6 glioma cells were cultured to near confluency in DMEM supplemented with 10% fetal calf serum at 37°C, and treated with forskolin (10 μM) plus dexamethasone (Dex) (1 μM) for 24 h before harvesting (final cell densities: Sertoli cells, 6×10^6 cells/dish; C6 glioma cells, 7×10^6 cells/dish). Values are means ± SE ($n = 5$)

4. DISCUSSION

Kilpatrick et al. [14] have reported that mouse testis contains a considerable amount of 1.45 kb ppEnk mRNA before postnatal day 16. We have observed a small amount of 1.5 kb ppEnk mRNA in rat testis before postnatal day 20 (unpublished), although the level was much lower than that of 1.9 kb ppEnk mRNA in the germ cells that appeared after postnatal day 30 [5]. These findings suggest that ppEnk mRNA in the testis during the early period of postnatal development arises from the Sertoli cells. However, testicular somatic cells expressing the ppEnk gene in vivo need to be identified by future studies (e.g. in situ hybridization histochemistry).

Sertoli cells possess high-affinity opiate-binding sites [15], through which β-endorphin, another opioid peptide produced by Leydig cells, modulates the functions of adjacent Sertoli cells as a paracrine regulator [16,17]. It is tempting to speculate that enkephalins and related peptides produced by Sertoli cells also modulate the Sertoli cell functions as an autocrine regulator.

Besides FSH, various neurotransmitters and hormones such as β-adrenergic agonists, muscarinic cholinergic agonists [18], opioid peptides [16], and α-melanocyte stimulating hormone [19] modulate the functions of Sertoli cells. Thus, Sertoli cells may represent a useful model system for studies on multifactorial regulation of ppEnk gene expression in the nervous system.

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