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Expression of pituitary adenylate cyclase activating polypeptide in the adult rat testis by *in situ* hybridization and immunohistochemistry

Phil-ok Koh*, Soo-dong Kwak

Institute of Animal Medicine, College of Veterinary Medicine Gyeongsang National University
**Department of Oral and Craniofacial Biological Sciences, University of Maryland, Baltimore, Maryland, USA*
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Abstract : Pituitary adenylate cyclase activating polypeptide (PACAP) was originally isolated from the ovine hypothalamus and stimulated cAMP production in anterior pituitary cells. It is known that PACAP stimulates cAMP accumulation and contributes to the spermatogenesis and steroidogenesis in rat testis. The principal aim of this study is to determinate the distribution of PACAP mRNA and protein in adult rat testis. For this study, we used *in situ* hybridization and immunohistochemistry techniques in adult rat testis. PACAP mRNA was stage specifically expressed in seminiferous tubules. Positive signals of PACAP mRNA were detected in the developing germ cells at stages III~VII of the epithelial cycle. The strongest signals of PACAP mRNA and protein were detected in round spermatids at stages V to early VII of the cycle. These results demonstrate that PACAP which is synthesised in the developing germ cells contributes to the spermatogenesis in rat testis. Thus, we suggest that PACAP plays a critical role in the function of testis.

Key words : PACAP, rat, testis

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) was originally isolated from the ovine hypothalamus and stimulated cAMP production in anterior pituitary cells[1]. PACAP has considerable homology with secretin, glucagon, vasoactive intestinal peptide (VIP), and growth hormone releasing hormone. PACAP exists in two biologically active forms, PACAP 38 and PACAP 27. PACAP 38 and PACAP 27 are derived from a precursor of 175 amino acid residues[2]. PACAP binds to three type receptors. PACAP type I receptor specifically binds to both PACAP 38 and PACAP 27, and binds VIP with only very low affinity. PACAP type II receptor has approximately equal high affinity for PACAP 38, PACAP 27, and VIP[3-5]. A third type of receptor binds PACAP and VIP with similar affinity, another member of the VIP peptide family, VIP2 receptor[6]. PACAP and its receptors have been found in the central nervous system and the peripheral tissue, including the hypothalamus, cortex, hippocampus, adrenal gland, testis, and ovary[7-12]. The presence of PACAP in hypothalamus, testis, and ovary suggests that PACAP has important roles in the reproductive system.

The highest concentrations of PACAP were detected in the testis[7,13]. PACAP stimulated cAMP accumulation and testosterone secretion in isolated adult rat Leydig cells[14-16]. In the previous studies, northern blot analysis showed that PACAP mRNA was stage specifically expressed in the rat testis. Furthermore, the highest amount of PACAP mRNA was detected at stages V to early VII of spermatogenesis[17,18]. As the further study, the present study was performed to evaluate the distribution of PACAP mRNA and protein in adult rat testis where spermatogenesis and steroidogenesis actively occur. Thus, we performed *in situ* hybridization and immunohistochemistry techniques in adult rat testis.

Materials and Methods

Animals and tissue preparation

Sprague-Dawley male rats (250~300 g) were maintained

Address reprint requests to Dr. Soo-Dong Kwak, College of Veterinary Medicine, Gyeongsang National University, Chinju 660-701, Korea.

at 25°C, and lighting (14 hr light : 10 hr dark), and allowed free access to food and water. For *in situ* hybridization and immunohistochemistry studies, animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) through the left cardiac ventricle. Testes were removed, fixed, and cryoprotected with 20% sucrose phosphate buffer for 24 hrs. Sections in 15 μm thickness were cut in a cryomicrotome, mounted on the probe-on plus-charged slides (Fisher Scientific, Pittsburgh, PA), and stored at -70°C until use. Sections from each testis were stained with hematoxylin and eosin for general morphological observation.

In situ Hybridization

All solutions were made with sterile water, and glassware was autoclaved to prevent contamination by RNase. *In situ* hybridization was carried out as described by Angerer *et al*[19]. The sections were dried, washed with 0.1 M PBS, treated with proteinase K, TE buffer, and acetylation solution. The sections were covered with prehybridization buffer containing 50% deionized formamide and incubated at 37°C for 1 hr. After removal of the prehybridization buffer, the sections were covered with the mixture containing the prehybridization buffer, 50 μg/m*l* yeast tRNA, 10 mM dithiothreitol, and ^{35}S-labeled PACAP cRNA probe. The sections were covered with cover glasses and incubated at 60°C for 24 hrs. ^{35}S-UTP labeled probe was prepared using *in vitro* transcription kit (Promega, Madison, WI). Antisense and sense cRNA probes were purified with a Sephadex G-50 nick column (Pharmacia Biotech, Uppsala, Sweden) and eluted with SET buffer containing 0.1% SDS, 1 mM EDTA, 10 mM Tris, and 10 mM DTT. Tissue sections were posthybridized in a posthybridization buffer. Following a wash in 4× SSC for 30 mins, the sections were then treated with ribonuclease A (50 μg/m*l*) at 37°C for 10 mins, washed twice in 2× SSC and 1× SSC, transferred to a wash buffer containing 0.1× SSC at 65°C for 30 mins, and dehydrated in alcohol solutions with ascending concentrations. The sections were exposed to β-max autoradiography X-ray film (Amersham, Uppsala, Sweden) for 4 days in light-tight cassettes at -70°C. They were dipped into NTB2 emulsion (1:1 dilution, Eastman Kodak Co., New York, NY), exposed at 4°C for 2 weeks, developed in Kodak D19 developer (1:1 dilution, Eastman Kodak Co.) at 15°C, and counter-stained with hematoxylin. The slides were observed under a dark and a bright field microscope, and then photographed. As a negative controls, hybridization was also carried out using sense strand cRNA probe.

Immunohistochemistry analysis

The sections were dried, washed with 0.1 M PBS, incubated in 0.3% H_2O_2 for 10 mins, and rinsed thoroughly with PBS. The sections were blocked with 1% normal goat serum in PBS at room temperature for 1 hr to suppress nonspecific binging of IgG, and then incubated with rabbit anti-PACAP 38 antiserum (1:1000 in PBS, Peninsula Laboratories Inc., Belmont, USA) at 4°C for 18 hrs in a humidified chamber. After washing with PBS, sections were incubated with biotin-conjugated goat anti-rabbit IgG (1:200 in PBS) at room temperature for 1 hr, followed by avidin-biotin-peroxidase complex for 1 hr from a Vector ABC Elite kit (Vector Laboratories Inc., Burlingame, USA). The sections were again washed with PBS, and incubated with diaminobenzidine tetrahydrochloride (Sigma chemical Co., St. Louis, MO) solution with 0.03% hydrogen peroxidase for 3 mins. As a negative control, normal serum was applied to primary antibody reaction in this experiment. The sections were observed under light microscope, and then photographed.

Results

PACAP mRNA was stage specifically detected in seminiferous tubules. Positive tubules of PACAP mRNA were detected in approximately one third of the cross section of the seminiferous tubules (Fig 1). We classified the stages I~X IV of the epithelial cycle within seminiferous tubules by Leblond *et al*[20] method. Positive signals of PACAP mRNA were observed in the developing germ cells, spermatogonia, and primary spermatocytes at stages III~VII of the epithelial cycle. The strongest signal of PACAP mRNA was detected in round spermatis at stages V to early VII of the cycle. However, no positive signals of PACAP mRNA were detected in Sertoli cells and Leydig cells (Fig 2). There were no detectable signals in negative control with a sense probe.

The distribution of PACAP protein was identified by immunohistochemistry technique. Positive signals of PACAP protein were detected in the developing germ cells and strongly detected in spermatids situated near the lumen of the seminiferous tubules. The strongest signals of PACAP were detected at stages V~VII of the epithelial cycle. Positive signals of PACAP were weakly detected in spermatogonia and primary spermatocytes.

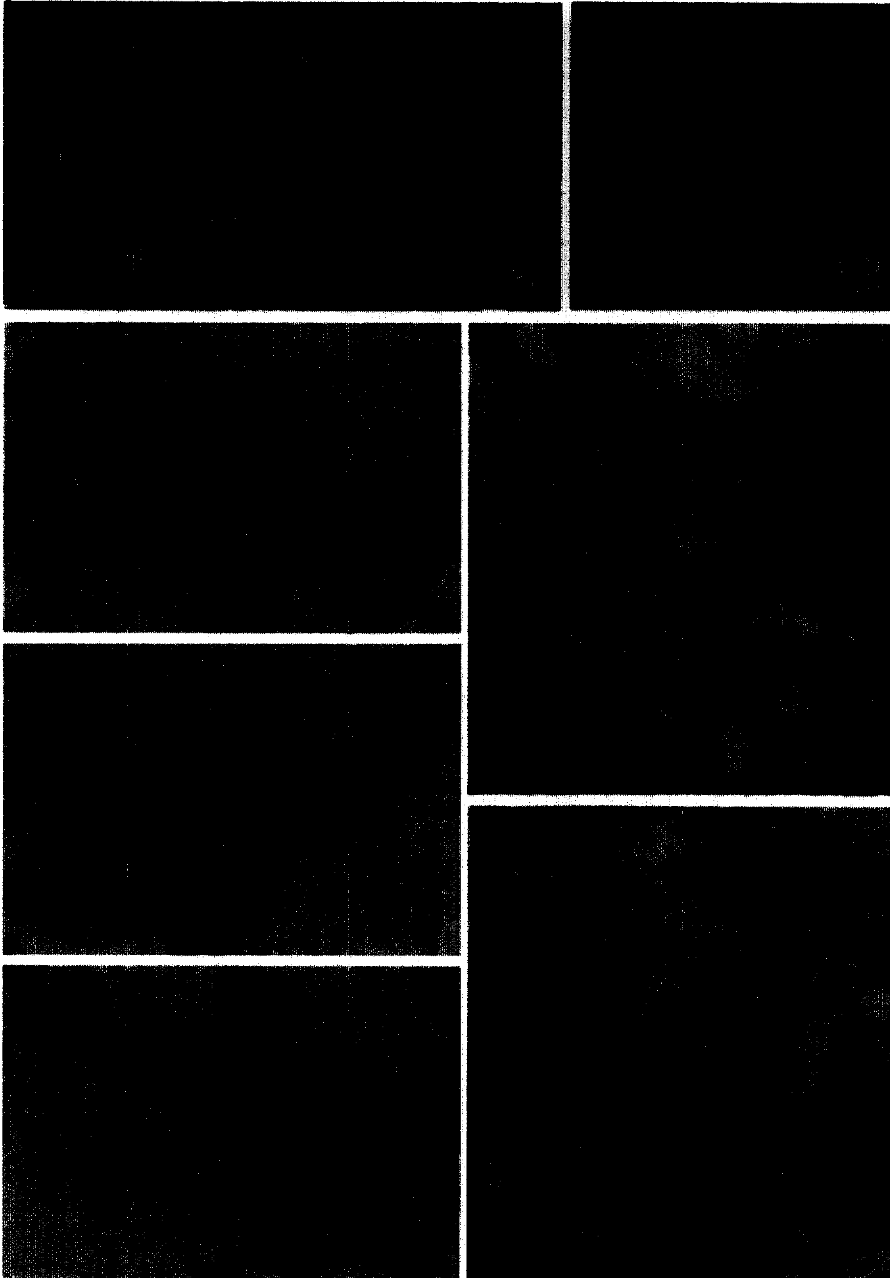

Fig 1. Dark-field photomicrographs for PACAP mRNA in rat testis by *in situ* hybridization. A, Positive signals were observed in seminiferous tubules at stages III~VII of the epithelial cycles. B, No positive signal were detected in negative control with a sense probe. Scale bar = 250 μm.

Fig 2. Bright-field photomicrographs for PACAP mRNA in rat testis by *in situ* hybridization. A and B, Intensive hybridization signals were observed in the round spermatids at the stage V~VII of the cycle. C, There were no detectable signals in the spermatids at the stage I, II, VIII~XIV. Arrows indicate positive cells of spermatids. Scale bar = 50 μm.

Fig 3. Localization of PACAP protein in rat testis by immunohistochemistry. A and B, Positive cells were detected in the developing germ cells. Intense PACAP was found in spermatids at the stages V~VII of the seminiferous tubules. Arrows indicate positive cells of spermatids. Scale bar = 50 μm.

However, no positive signals of PACAP were found in mature spermatocytes, testicular spermatozoa, and epididymal spermatozoa (Fig 3).

Discussion

The previous studies reported that the highest concentration of PACAP 38 was found in the testis and the total amount of PACAP in both testes exceeded its content in the whole brain[7]. PACAP was stage specifically expressed in the seminiferous tubules, the highest amount of PACAP was detected at stages V~VII of spermatogenesis by northern blot analysis[17]. In this study, we showed the localization of PACAP mRNA and protein in adult rat testis by *in situ* hybridization and immunohistochemistry. PACAP mRNA and protein were expressed in developing germ cells within seminiferous tubule at stage III~VII of the epithelial cycle in adult rat testis and strongly expressed in the spermatids during their early developmental stage, at stages V~VII of spermatogenesis. It is known that these stages are considered as the onset of meiosis at spermatocytes[21]. Furthermore, the developing germ cells contribute to the regulation of spermatogenesis by direct cell to cell interactions and by secreting diffusible paracrine factors[22-24]. Also, PACAP stimulates cAMP production in spermatocytes. The present study demonstrates that PACAP which is produced in spermatides actives spermatocytes. Thus, our results suggest that PACAP regulates spermatogenesis through the maturation of germ cells.

PACAP stimulated the cAMP accumulation and steroidogenesis in the various tissues[15,16,25-27]. Especially, PACAP was detected in granulosa cells and corpus luteum of the rat ovary and significantly stimulated the cAMP accumulation and progesterone production in these cells[25-28]. Also, PACAP stimulated the testosterone production in rat Leydig cells[15,16]. However, in this study, there were no detectable signals of PACAP mRNA and protein in Sertoli cells and Leydig cells. Shivers *et al*[9] reported only background labeling or low number of binding sites for PACAP in Sertoli cells. Monts *et al*[29] detected the presence of PACAP type I receptor in Leydig cells. Also, Rossato *et al*[16] demonstrate that PACAP 38 stimulates testosterone secretion in isolated adult Leydig cells through the interaction with a PACAP type I receptor. In these results, we can suggest that PACAP acts as a paracrine regulator for the steroidogenesis in Leydig cells.

In the present study, we showed that PACAP mRNA and protein were stage specifically expressed in the seminiferous tubules. The expression of PACAP in the developing germ cells supports the fact that PACAP contributes to the testicular functions, spermatogenesis and steroidogenesis. In conclusion, we suggest that PACAP plays a critical role in the function of testis.

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In situ hybridization법과 면역조직화학적법을 이용한 성숙한 흰쥐고환에서의 pituitary adenylate cyclase activating polypeptide의 발현

고필옥* · 곽수동

경상대학교 수의과대학 동물의학연구소
*미국 Maryland 의과대학교, Oral and Craniofacial Biological Sciences과
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국문초록 : Pituitary adenylate cyclase activating polypeptide (PACAP)는 양의 뇌하수체에서 처음 분리되었고, 뇌하수체 전엽세포의 cAMP의 생성을 자극하며, 흰쥐고환의 정자형성과 steroid 호르몬 형성에 관련한다고 알려져 있다. 이 연구는 성숙한 흰쥐의 고환에서 PACAP mRNA와 그 단백질의 분포를 조사하여 아래와 같은 결론을 얻었다. PACAP mRNA와 그 단백질은 흰쥐의 정세관에서 정자세포의 생성단계에 따라 특이적으로 발현되었다. 이들은 정세관의 발달단계 중 III~VII 기의 정자세포에서 발현되었고, 특히 V 기에서 초기 VII 기의 원형의 정자세포에서 가장 강하게 발현되었다. 이러한 결과는 흰쥐고환의 발달단계에 있는 정자세포에서 합성된 PACAP이 정자형성에 관련된다는 것을 나타내므로, PACAP이 고환의 기능에 중요한 역할을 하는 것을 암시한다.

중심어 : PACAP, 흰쥐, 고환