General and Comparative Endocrinology 168 (2010) 256–261

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Pituitary adenylate cyclase-activating polypeptide and its receptor PAC_1 in the testis of *Triturus carnifex* and *Podarcis sicula*

Marisa Agnese [a,*], Salvatore Valiante [a], Francesco Angelini [b], Vincenza Laforgia [a], Piero Andreuccetti [a], Marina Prisco [a,**]

[a] Department of Biological Sciences, University of Naples "Federico II", Via Mezzocannone 8, 80134 Napoli, Italy
[b] Department of Structural and Functional Biology, University of Naples "Federico II", Via Cinthia, 80126 Napoli, Italy

ARTICLE INFO

Article history:
Received 15 November 2009
Revised 18 March 2010
Accepted 19 March 2010
Available online 23 March 2010

Keywords:
PACAP
PAC_1R
Spermatogenesis
Steroidogenesis
Triturus carnifex
Podarcis sicula

ABSTRACT

The pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the glucagon-related family that occurs in two amidated forms with 38 (PACAP38) and 27 (PACAP27) amino acids. First discovered in the brain, it was then localized in several peripheral tissues of mammals, including the testis. However, current knowledge of the expression and function of PACAP and its receptor PAC_1 in the reproductive system of non-mammalian vertebrates, and particularly in the testis, is still limited. The aim of this work was to study the presence of PACAP and its receptor PAC_1 in the testis of two non-mammalian vertebrates during the breeding season: the crested newt *Triturus carnifex* and the wall lizard *Podarcis sicula*. The expression and distribution of this neuropeptide and its receptor PAC_1 were investigated by using *in situ* hybridization and immunohistochemistry techniques. Our results demonstrated that PACAP and its receptor PAC_1 were highly represented in the testis of these two species. In particular, we showed that they are present within some germ cells and that PACAP, unlike in mammals, is expressed also in the somatic cells (Sertoli and Leydig cells) of the testis of these two non-mammalian vertebrates, suggesting that this neuropeptide is involved in the hormonal control of spermatogenesis and steroidogenesis.

1. Introduction

The pituitary adenylate cyclase-activating polypeptide (PACAP) is a bioactive neuropeptide and a member of the growth hormone-releasing hormone (GHRH)/vasoactive intestinal peptide (VIP)/glucagon family (Arimura, 1998; Sherwood et al., 2000; Vaudry et al., 2000, 2009). Its distribution has been extensively studied since it was first isolated from ovine hypothalamic tissues based on its ability to stimulate *in vitro* adenylate cyclase (Miyata et al., 1989). PACAP is present in two biologically active forms, a longer form consisting of 38 amino acids (PACAP38) and a shorter one (PACAP27) containing the 27 N-terminal amino acids of PACAP38 (Miyata et al., 1989, 1990). Three PACAP receptors have been identified: PAC_1, which binds PACAP exclusively, $VPAC_1$ and $VPAC_2$, which bind PACAP and VIP with equal affinity. All are G protein-coupled receptors with a classical structure of seven transmembrane domains (Harmar et al., 1998); they each possess a different structure, specificity and tissue distribution, and activate different intracellular signaling pathways (Dickson and Finlayson, 2009).

The presence of both PACAPs and their receptors in different vertebrate organs and tissues suggested that PACAP can be considered a pleiotropic neuropeptide, that acts as a hypophysiotropic hormone, a neuromodulator, a vasoregulator of secretion and a regulatory factor of reproduction (for reviews, see Arimura, 1998; Sherwood et al., 2000; Vaudry et al., 2000). In this regard it is worth noting that high concentrations of PACAPs are evident in the testis, where the PACAP38 concentration is about two times greater than that of the whole brain (Arimura et al., 1991). The presence and function of PACAP in mammalian testis have been widely studied; in the rat testis, PACAP was reported to be present in mitotic and meiotic germ cells and in early spermatids, but not in mature spermatids, spermatozoa and in somatic cells, i.e., Sertoli and Leydig cells (Shioda et al., 1994; Hannibal and Fahrenkrug, 1995; Romanelli et al., 1997), whereas its receptors were found in germ (Shivers et al., 1991), Leydig (Romanelli et al., 1997) and Sertoli cells (Heindel et al., 1992). In this regard it was hypothesized that PACAP could activate protein synthesis in spermatocytes, inhibit synthesis in spermatids *in vitro* (West et al., 1995), stimulate cAMP accumulation and secretion of lactate, estradiol and inhibin in Sertoli cells (Heindel et al., 1992), as well as induce cAMP accumulation and testosterone secretion in Leydig cells in a dose-dependent

* Correspondence to: M. Agnese, Department of Biological Sciences, Evolutionary and Comparative Biology Division, Via Mezzocannone 8, 80134 Napoli, Italy.
** Correspondence to: M. Prisco, Department of Biological Sciences, Evolutionary and Comparative Biology Division, Via Mezzocannone 8, 80134 Napoli, Italy.
E-mail addresses: marisa.agnese@unina.it (M. Agnese), maprisco@unina.it (M. Prisco).

0016-6480/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2010.03.016

manner (Romanelli et al., 1997; Rossato et al., 1997; El-Gehani et al., 1998; Vaudry et al., 2000). Although the physiological significance of PACAP and its receptors remains unknown in the testis, it has been proposed that PACAP acts as an intragonadal regulator in mammalian reproduction (Moretti et al., 2002).

Information regarding the roles of PACAP in non-mammalian vertebrates testis is lacking, except for the *in vitro* investigation by Gobbetti and Zerani (2002) showing that PACAP induces testicular testosterone synthesis through prostaglandin mediation in *Triturus*, and for the findings of Huang et al. (2009) concerning expression and regulation of PACAP in tilapia gonads.

The aim of this work was to study the expression of PACAP and its receptor PAC_1 in the testis of two non-mammalian vertebrates: the crested newt *Triturus carnifex* and the wall lizard *Podarcis sicula*, that are both seasonal breeders, characterized by a different organization of the testis: cystic in *Triturus* (Galgano, 1944) and tubular in *Podarcis* (Galgano and D'Amore, 1953; Angelini et al., 1979). Our investigation was performed during the breeding stage: in this period the different stages of male germ cells differentiation are all present at the same time in the two testes.

By using *in situ* hybridization and immunohistochemistry, we showed that PACAP and PAC_1 are expressed within somatic cells involved in testicular steroidogenesis, as well as in germ cells, thus suggesting that PACAP has an active role also in the male reproductive processes of non-mammalian vertebrates.

2. Materials and methods

2.1. Animals

Sexually mature males of *T. carnifex* and *P. sicula* were collected in the region of Campania (southern Italy) during the breeding season (February–March for *Triturus* and May for *Podarcis*). *Triturus* males were maintained in aquaria under conditions of natural photoperiod and temperature, and fed *ad libitum* with liver; *Podarcis* males were maintained in a soil-filled terrarium and fed *ad libitum* with *Tenebrio* larvae. The experiments were approved by institutional committees (Ministry of Health) and organized to minimize the number of animals used. Newts were anesthetized by hypothermia, chilling them in chipped ice; lizards were sacrificed by decapitation after deep anesthesia with ketamine hydrochloride (Parke-Davis, Berlin, Germany) 325 μg/g of body weight. The testes were quickly removed, fixed for 24 h in Bouin's solution and then dehydrated and embedded in paraffin wax. After microtome sectioning to 7 μm, sections were placed on superfrost or polylysine glass slides (Menzel-Glaser, Braunschweig, Germany). Consecutive sections were utilized for *in situ* hybridization and immunohistochemistry procedures.

2.2. In situ hybridization

All solutions used in *in situ* hybridization were made with bidistilled water treated with 0.1% diethylpyrocarbonate (DEPC) (Sigma, St. Louis, MO) to avoid RNase contamination and then autoclaved. Lizard *PACAP* cDNA (Valiante et al., 2007) was ligated in PsPT18 plasmid (Roche Diagnostics, Mannheim, GE); JM109 cells were then transfected overnight at 37 °C. Plasmids containing the *PACAP* cDNA insert were recovered with an Ultraclean miniplasmid kit (MoBIO labs, Carlsbad, CA) following the manufacturer's instructions, and an *in vitro* transcription reaction was carried out to synthesize 200 bp digoxigenin labeled sense and antisense RNA probes (Roche). *In situ* hybridization procedure was performed as described elsewhere (Valiante et al., 2004). Briefly, slides were hybridized overnight at 60 °C in the hybridization buffer, containing 50% deionized formamide, SSC 5×, Denhardt solution 1×, denatured salmon sperm DNA 100 μg/ml, tRNA 100 μg/ml, and 20% dextran sulfate added with 2 ng/μl of lizard *PACAP* sense or antisense riboprobes. After post-hybridization washes in 0.5× SSC and 20% formamide, slides were treated with RNase A 10 μg/ml and then with 2% blocking reagent (Roche), 10% normal sheep serum in 100 mM maleic acid buffer pH 7.4. To reveal digoxigenin, anti-DIG alkaline phosphatase conjugated antibody (Roche) was used and BM Purple (Roche) colorimetric reaction was performed following the manufacturer's instruction. *In situ* hybridization signal was analyzed with Axioskop System (Zeiss, Oberkochen, Germany).

2.3. Immunohistochemistry

Slides were immersed in 0.1 M citrate buffer, pH 6.0, in a microwave oven (2 × 10′) for antigen retrieval. Sections were rinsed with 0.5% H_2O_2 to inactivate endogenous peroxidases and with normal goat serum (Pierce, Rockford, IL) to reduce non-specific background. Rabbit anti-PACAP27 antibody (Phoenix Pharmaceuticals, Belmont, CA, USA; this antibody shows 0% cross-reactivity with PACAP38) diluted 1:1000 or anti-PAC_1R (Santa Cruz Biotechnology) diluted 1:50 in normal goat serum was applied overnight at 4 °C. The reaction was revealed with a biotin-conjugated goat anti-rabbit secondary antibody and an avidin–biotin–peroxidase complex (ABC immunoperoxidase Kit, Pierce), using DAB as chromogen. Sections were counterstained with Mayer's hemallum. Negative controls were carried out by omitting primary antibody. Immunohistochemical signal was analyzed with Axioskop System (Zeiss, Oberkochen, Germany).

3. Results

3.1. Cellular localization of PACAP transcript

Analysis of *PACAP* mRNA distribution by *in situ* hybridization revealed a wide expression of the *PACAP* gene in the testis of *T. carnifex* and *P. sicula*.

3.1.1. Triturus carnifex testis (Fig. 1a–c)

In the newt testis the signal was detected in mitotic and meiotic germ cells, i.e., spermatogonia (Fig. 1a) and spermatocytes I (Fig. 1b); no hybridization signal was present at the level of spermatozoa (Fig. 1c). A positive signal also occurred within somatic cells, i.e., Sertoli and Leydig cells. The latter were stained only if located among cysts containing spermatogonia and spermatocytes I (Fig. 1a and b); no signal was evident in Leydig cells located among cysts containing spermatozoa (Fig. 1c). Sertoli cells showed the same hybridization pattern of Leydig cells (Fig. 1a and b). Control sections incubated with *PACAP* sense riboprobe showed no signal (data not shown).

3.1.2. Podarcis sicula testis (Fig. 1d and inset)

In the lizard testis, mRNA was expressed in all the stages of spermatogenesis, and in particular in the last stages (spermatids and spermatozoa), where quite a strong hybridization signal was evident (Fig. 1d). A positive signal occurred also within Sertoli and Leydig cells (Fig. 1d). Control sections, performed with sense riboprobe, were not labeled (Fig. 1d, inset).

3.2. Cellular localization of PACAP protein

PACAP protein localization in *T. carnifex* and *P. sicula* testis was performed by immunolocalization experiments.