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Role of PACAP on testosterone and 17β-estradiol production in the testis of wall lizard *Podarcis sicula*

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ABSTRACT

Pituitary adenylate cyclase-activating peptide (PACAP) is a neuropeptide that in mammalian testis is involved in the control of testosterone and 17β-estradiol synthesis. A similar involvement was recently postulated in the testis of a nonmammalian vertebrate, the wall lizard *Podarcis sicula*. Indeed, we reported the presence of PACAP and its receptors throughout the reproductive cycle within both germ and somatic cells. Now, we investigated the effects of PACAP on steroidogenesis in significant periods of *Podarcis* reproductive cycle: winter stasis, reproductive period and summer stasis. Using different in vitro treatments, in the absence or presence of receptor antagonists, we demonstrated that in *P. sicula* testis PACAP is involved in the control of testosterone and 17β-estradiol production. In particular we demonstrated that treatment with PACAP induced a testosterone increase only in stasis periods (winter and summer stasis); differently they induced a 17β-estradiol production in all periods analyzed (summer stasis, winter stasis and reproductive period).

1. Introduction

Pituitary adenylate cyclase-activating peptide (PACAP) is a neuropeptide highly conserved in sequence, isolated firstly from the ovine hypothalamus for its ability to stimulate in vitro adenylate cyclase (Miyata et al., 1989). It is 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) which includes glucagon, GLP-1 (Glucagon-Like-Peptide-1), GLP-2, GIP (Glucose-Dependent-Insulinotropic Polypeptide), GHRH, PHM (Peptide Histidine–Methionine), PACAP, secretin and VIP (Sherwood et al., 2000). PACAP is present in two biologically active forms, PACAP27 and PACAP38, of 27 and 38 amino acids, respectively (Miyata et al., 1989, 1990). Three PACAP receptors have been identified: PAC_1, which binds exclusively PACAP; $VPAC_1$ and $VPAC_2$, which bind PACAP and VIP with the same affinity. The presence of PACAP and its receptors in different vertebrate organs and tissues has suggested that PACAP can be considered as a pleiotropic neuropeptide, that may act as hypophysiotropic hormone, neuromodulator, vasoregulator of secretion, and also as a regulatory factor for reproduction (for reviews, see Arimura, 1998; Sherwood et al., 2000; Vaudry et al., 2000). At this regard, it is worth noting that in the mammalian testis PACAP concentration is about two fold greater than that of the whole brain (Arimura et al., 1991). Immunohistochemical investigation showed that in rat testis PACAP occurs in spermatids and in sperm acrosome, but not in somatic cells, i.e. Sertoli and Leydig cells (Shioda et al., 1994; Hannibal and Fahrenkrug, 1995; Romanelli et al., 1997; Tanii et al, 2011), while, in humans it occurs also in spermatogonia and round spermatids (Nakamura et al., 2014). Finally, in vitro experiments showed that in mammalian testis PACAP induces testosterone and 17β-estradiol secretion in a dose-dependent manner in Leydig and Sertoli cells (Heindel et al., 1992; Romanelli et al., 1997; Rossato et al., 1997), demonstrating that PACAP has a significant role in the control of mammalian spermatogenesis.

The first information concerning the involvement of PACAP in the testis activity of a nonmammalian vertebrate was reported by Gobbetti and Zerani (2002), who demonstrated that in *Triturus carnifex* PACAP induces testosterone synthesis by prostaglandin mediation. More recently, we reported the presence of PACAP and its receptors in the testis of *Torpedo marmorata* (Agnese et al., 2015), *T. carnifex* (Agnese et al., 2010), and *P. sicula* (Agnese et al., 2010; Rosati et al., 2014a,b). In particular, the studies performed in *Podarcis* during male reproductive cycle (Agnese et al., 2010; Rosati et al., 2014a,b), showed that lizard PACAP is synthesized in the testis, as in mammals (Li and Arimura, 2003). Furthermore, we demonstrated that PACAP, PAC_1 and $VPAC_2$ are widely represented in germ and somatic cells (Agnese et al., 2010; Agnese et al., 2014b,c; Rosati et al., 2014a,b), while $VPAC_1R$ occurs only within Leydig cells and spermatids (Agnese et al., 2014b,c). These data suggested that PACAP is involved in the control of *P. sicula* testicular activity, including spermatogenesis and steroidogenesis (Agnese et al., 2010; Agnese et al, 2014b; Rosati et al., 2014a,b).

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The aim of this work is to assess the involvement of PACAP on steroidogenesis of *Podarcis* by testis cultures after PACAP addition in the absence or presence of its receptor antagonists. We evaluated the effects of PACAP at physiological concentration, alone or with the antagonists of PAC_1 (M65 Antagonist), $VPAC_1$ ($VPAC_1$ Receptor Antagonist) and $VPAC_2$ (PG99-465 Antagonist) receptors (Gourlet et al., 1997; Moreno et al., 2000) on the steroidogenesis, particularly on the testosterone and 17β-estradiol production. Such hormones have a pivotal role in the control of *Podarcis* spermatogenesis: testosterone is essential in the reproductive period, and 17β-estradiol regulates the stasis periods (Angelini and Botte, 1992). The investigations were performed in three significant phases of wall lizard reproductive cycle: the summer and winter stasis, and the reproductive period. Indeed, the three phases are characterized by a different seminiferous tubule organization, and a different hormonal profile: in the summer stasis, when the highest 17β-estradiol plasmatic level is recorded, the spermatogenesis is arrested and the tubules contain only spermatogonia; in the winter stasis, when the hormonal profile is similar to summer stasis, the spermatogenesis is blocked but the seminiferous tubules contain all the spermatogenic stages (including spermatozoa) resulting from the previous resumption occurred in November; in the reproductive period, the testosterone titre is prevalent and the spermatogenesis finishes with the release of functional spermatozoa that are utilized for reproduction. Our results demonstrate that in *P. sicula* PACAP, as previously reported for VIP (Rosati et al., 2015), is involved in the control of testosterone and 17β-estradiol production in time- and dose-dependent ways.

2. Materials and methods

2.1. Animals

Sexually mature males of *P. sicula* were collected in Campania (Southern Italy) during different periods of the reproductive cycle: January for winter stasis, May for reproductive period and July for summer stasis. We used 10 animals for each period. The animals were collected in the same year (2013).

Males were maintained in a soil-filled terrarium and fed ad libitum with *Tenebrio molitor* larvae. The experiments were approved by institutional committees (Ministry of Health, Italy) and organized to minimize the number of animals used. The animals were sacrificed by decapitation after deep anesthesia with ketamine hydrochloride (Parke-Davis, Berlin, Germany) 325 pg/g of body weight (Valiante et al., 2007; Valiante et al., 2008). Sexual maturity of each animal was determined by morphological parameters and by histological analysis (Agnese et al., 2014a,b,c; Rosati et al., 2014a,b).

After the dissection, part of testes was fixed in Bouin's solution, dehydrated in a graded ethanol series and embedded in paraffin wax (Del Giudice et al., 2011a,b, 2012; Prisco et al., 2009a,b); then they were sectioned and stained with Mayer's hematoxylin and eosin (Agnese et al., 2012, 2013a,b,c) to evaluate the conditions of the testis before the in vitro treatments (non-treated testis). The remaining part of testes was used for cultures.

2.2. Testis cultures

Testis cultures procedure was performed as described elsewhere (Rosati et al., 2015). First, we performed a preliminary investigation on animals collected in May to assess the PACAP38 action on steroidogenesis. We tested three PACAP concentrations (10^{-8} M M, 10^{-7} M and 10^{-6} M) and three different incubation times (30, 60 and 120 min). Testis fragments (60 mg each) were mixed and randomly assigned to each well (two fragments for well), in 24-well plates at 25 °C with 5% CO_2. The control fragments were treated with medium alone. Each treatment was performed in quadruplicate: it means that testis fragments of different animals, once mixed and randomly assigned in four wells in the same plate, were exposed to the same treatment.

After the preliminary investigation and basing on its results, we performed the further analysis choosing 10^{-7} M PACAP in concentration (physiological concentration) and 2 h for incubation time, as at this time the maximum hormone secretion was recorded, independently from the concentration used. Now, using the receptor antagonists, we investigated the receptors involved in testis steroidogenesis in reproductive period and in winter and summer stasis. Testis fragments were mixed and randomly assigned, in 24-well plates at 25 °C with 5% CO_2 for 2 h. At the end of 2 h, the medium was collected (zero time) and replaced with fresh medium, 2 ml for well, containing PACAP in the absence or presence of receptor antagonists, according to the following scheme: treatment 1: medium alone (control); treatment 2: 10^{-7} M PACAP; treatment 3: 10^{-7} M PACAP in the presence of 10^{-6} M receptor antagonist $VPAC_1$ "VIP 1 Receptor Antagonist" (VIP1 Antagonist) (Phoenix Pharmaceuticals, Inc.) and 10^{-6} M receptor antagonist $VPAC_2$ "PG99-465" (Bachem); treatment 4: 10^{-7} M PACAP in the presence of 10^{-6} M receptor antagonist PAC_1 "M65" (Bachem) and 10^{-6} M receptor antagonist $VPAC_2$ "PG99 465" (Bachem); treatment 5: 10^{-7} M PACAP in the presence of 10^{-6} M receptor antagonist PAC_1 "M65" (Bachem) and 10^{-6} M receptor antagonist $VPAC_1$ "VIP 1 Receptor Antagonist" (VIP1 Antagonist) (Phoenix Pharmaceutical); treatment 6: 10^{-7} M PACAP in the presence of three antagonists at 10^{-6} M. To facilitate the blocking of receptors, the antagonists were dissolved in the medium 1 h before the PACAP supplement and at the 10^{-6} M concentration, 10-fold higher than PACAP concentration.

After 2 h of treatment, specimens were fixed in Bouin's solution for 24 h, dehydrated through an ascending series of alcohols and embedded in paraffin wax. 7 μm thick sections were stained with Mayer's hematoxylin and eosin and observed by light microscopy. The media, once collected were stored at −20 °C and used for the hormone assays later. In each experiment we used 20 wells for treatment.

2.3. Hormonal assays

The levels of testosterone and 17β-estradiol were determined using ELISA (Enzyme-linked immunosorbent assay) assays (DIAMETRA) as previously described in this species (Raucci and Di Fiore, 2009; Raucci et al., 2005). For testosterone, the limit of detection for sensitivity was 0.075 ng/ml with an analytical range of 0.2 to 16 ng/ml and an incubation time of 60 + 15 min with an intra-assay variability less than 5.8% and an inter-assay variability less than 10.5% (Raucci and Di Fiore, 2009). For 17β-estradiol, the limit of detection for sensitivity was 8.7 pg/ml with an analytical range of 20–200 pg/ml and an incubation time of 120 + 30 min with an intra-assay variability less than 9% and an inter-assay variability less than 10% (Raucci et al., 2005).

Results were analyzed using GraphPad 5.0 software (San Diego, California); statistical analysis was carried out by ANOVA test with Bonferroni's correction; p value < 0.05 was considered statistically significant.

3. Results

3.1. Hormonal assays

3.1.1. PACAP treatment

During the reproductive period, in *P. sicula* testis cultures PACAP treatment determined a time- and dose-dependent increase of testosterone (Table 1A) and 17β-estradiol (Table 1B) titres compared to the controls. These results let us select the ideal PACAP concentration (10^{-7} M) and time exposure (120 min) for our following experimental procedures.

3.1.2. PACAP and PACAP receptor antagonist treatment

3.1.2.1. Winter stasis. During winter stasis, PACAP alone, PACAP/VIP1 Antagonist/PG99-465 Antagonist, PACAP/M65 Antagonist/PG99-465