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RESEARCH ARTICLE

Sex Steroid Hormone Secretion in the Wall Lizard *Podarcis sicula* Testis: The Involvement of VIP

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ABSTRACT

Vasoactive intestinal peptide (VIP) is a pleiotropic neuropeptide involved in different functions, including testosterone synthesis. Recently, we reported the presence of VIP in the testis of *Podarcis sicula*, throughout the reproductive cycle. Now, we investigated the effects of the VIP on steroidogenesis in significant periods of the *Podarcis* reproductive cycle: winter stasis, reproductive period, and summer stasis. Using VIP treatments in testis culture in absence or presence of receptors antagonists, we demonstrated for the first time that in *P. sicula*, VIP is involved not only in testosterone synthesis, as in mammals, but in 17β-estradiol synthesis too. *J. Exp. Zool. 9999A:XX–XX, 2015.* © 2015 Wiley Periodicals, Inc.

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Vasoactive intestinal peptide (VIP) is a 28-amino acid peptide highly conserved in sequence, isolated and characterized firstly from porcine intestine for its ability to induce vasodilatation (Said and Mutt, '70). Then, VIP has been identified for the high similarity of primary and secondary structures as a member of the glucagon-secretin superfamily, which includes PACAP, secretin, glucagon-like peptide-1, growth-hormone-releasing factor 1–29, growth-hormone-releasing hormone, and helodermin (Said and Mutt, '70). As all the precursors of the glucagon-secretin family members, the VIP precursor (prepro-VIP) contains a sequence encoding an additional biologically active peptide, named peptide histidine isoleucine in non-mammalian vertebrates and peptide histidine methionine in mammals (Dickson and Finlayson, 2009). VIP acts through two G protein-coupled receptors, $VPAC_1R$ and $VPAC_2R$, two members of the 7-transmembrane domain receptors family, which activate the pathways of adenylate cyclase, phospholipase C, and phospholipase D (Dickson and Finlayson, 2009). VIP is a pleiotropic neuropeptide as it is involved in different functions, including the vaso-regulation, the immunoregulation, and the neuromodulation of numerous brain districts including cerebral cortex, hippocampus, amygdala, suprachiasmatic nucleus, hypothalamus, and pituitary (Köves et al., '91; Mikkelsen and Fahrenkrug, '94; Acsády et al., '96; Fahrenkrug and Hannibal, 2004; Dickson and Finlayson, 2009). Moreover, VIP is also involved in the control of reproduction (Sherwood et al., 2000; Vaudry et al., 2009); indeed, its presence has been reported in the reproductive organs and, in particular, within the nerve fibers of mammalian

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testis (Vaudry et al., 2009). Furthermore, in vitro experiments showed that VIP modulates the testosterone synthesis within Leydig cells (Heindel et al., '92; El-Gehani et al., '98a,b,c) by the interaction with $VPAC_1R$ and $VPAC_2R$, and spermatocytes by $VPAC_2R$ (Hueso et al., '89; Krempels et al., '95; Csaba et al., '97). The involvement of VIP in mammalian steroidogenesis is strengthened by the observation that VIP-deficient male rats are characterized by a low testosterone synthesis and an altered organization of the testis (Lacombe et al., 2007). More recently, the presence of VIP and its receptors has been reported in the testis of two non-mammalian vertebrates: the cartilaginous fish *Torpedo marmorata* (Agnese et al., 2012) and the wall lizard *Podarcis* sic*ula* (Agnese et al., 2014a,b). In particular, in *P.* sic*ula*, the distribution of the VIP/VPACR system was reported during all the phases of spermatogenic cycle, demonstrating that in lizards, differently from mammals, VIP is synthesized within the testis in both germ and somatic cells, where $VPAC_2R$ is present too. Differently, $VPAC_1R$ was always localized only within Leydig cells and spermatids. On the basis of VIP/VPACR system distribution, it has been hypothesized that in *Podarcis*, VIP is involved in the spermatogenesis and steroidogenesis (Agnese et al., 2014b).

The aim of this work is to assess the involvement of VIP on steroidogenesis of *Podarcis* by testis cultures after VIP administration in absence or presence of its receptors antagonists. In particular, we evaluated the effects of VIP at physiological concentration, alone or together with antagonists of $VPAC_1$ and $VPAC_2$ receptors, on the synthesis of the two hormones having a pivotal role in the control of *Podarcis* spermatogenesis: testosterone, essential in the reproductive period, and 17β-estradiol, regulating the stasis periods (Angelini and Botte, '92). The investigations have been performed in three significant phases of the reproductive cycle of this lizard: the winter and summer stasis, and the reproductive period. Both the stasis periods are characterized by the same low testicular activity but a different morphological organization: in the winter stasis the seminiferous tubules are constituted by germ cells from spermatogonia to spermatozoa, whereas in the summer stasis, the tubules are characterized by the presence of spermatogonia alone. Finally, in the reproductive period, when mating occurs, the testis shows seminiferous tubules characterized by all different cytotypes that are distributed from the basal membrane toward the lumen (Angelini and Botte, '92).

MATERIALS AND METHODS

Animals

Sexually mature males of *P.* sic*ula* 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, 2008). Sexual maturity of each animal was determined by morphological parameters and by histological analysis (Agnese et al., 2014a,b; Rosati et al., 2014b).

Part of testes was fixed in Bouin's solution, dehydrated in a graded ethanol series and embedded in paraffin wax to evaluate the conditions of the testis before the in vitro treatments (no treated testis); after the embedding, specimens were sectioned and stained with Mayer's hematoxylin and eosin. The remaining part of testes was used for cultures.

Testis Cultures

All solutions were filtered through 0.22 μm filter, autoclaved and sterilized under UV over night. As soon as taken, the testes of *P.* sic*ula* were washed in sterile cold physiological solution for reptiles (NaCl 0.75%), cut and then transferred in HAM-F10 medium (Sigma, Milano, Italy) containing 20 mM L-glutamine (Invitrogen, San Giuliano Milanese (MI), Italy), 7% FBS (Invitrogen), 100 U/mL penicillin (Invitrogen), 100 g/mL streptomycin (Invitrogen), 40 g/mL gentamicin (Invitrogen), and 20 mM Hepes (Sigma). First, we conducted a preliminary investigation on the testis of animals collected in May to assess the VIP action on steroidogenesis. We tested three VIP concentrations (10^{-8}M, 10^{-7}M, and 10^{-6}M) and three different times (30, 60, and 120 min). Testis fragments (60 mg each) were mixed and randomly assigned to each well (two slices for well), in 24-well plates at 25°C with 5% CO_2. The control slices were treated with medium alone. Each treatment was performed in quadruplicate, that is, 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, using the receptor antagonists, we investigated the receptor pathways 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 hr. This time was chosen on the basis of preliminary experimental tests, which showed that at 2 hr of time treatment, the maximum hormone secretion was recorded, independently from the concentration used. So, we used this incubation time and 10^{-7}M VIP (physiological concentration) for all the following treatments. At the end of the 2 hr, the medium was collected (zero time) and replaced with fresh medium, 2 mL for well, containing VIP in absence or presence of receptors antagonists, according to the following scheme: treatment 1: medium alone (control); treatment 2: 10^{-7}M VIP; treatment 3: 10^{-7}M VIP in presence of 10^{-6}M receptor antagonist $VPAC_1$ "VIP 1

receptor antagonist" (VIP1 Antagonist) (Phoenix Pharmaceuticals, Inc., Karlsruhe, Germany); treatment 4: 10^{-7}M VIP in presence of 10^{-6}M $VPAC_2$ receptor Antagonist "PG99-465" (Bachem, Bubendorf, Switzerland); treatment 5: 10^{-7}M VIP in presence of both antagonists at 10^{-6}M. To facilitate the blocking of receptors, the antagonists were dissolved in the medium 1 hr before the VIP supplement. Each treatment was performed in quadruplicate. After 2 hr of treatment, one slice was stored at −80°C and the other was fixed in Bouin's solution for 24 hr, dehydrated through an ascending series of alcohols and embedded in paraffin wax. Seven micrometer 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.

Hormonal Assays

The levels of testosterone and 17β-estradiol were determined using enzyme-linked immunosorbent assay (ELISA; DIAMETRA, Segrate - Milano, Italy) as previously described in this species. For testosterone, the limit of detection for sensitivity was 0,075 ng/mL with an analytical range of 0.2–16 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, CA, USA); statistical analysis was carried out by ANOVA test with Bonferroni's correction; p-value < 0.05 was considered statistically significant.

RESULTS

Hormonal Assays

VIP Treatment. Table 1 depicts the effects of VIP administration at different concentration sand for different times on testosterone (A) and 17β-estradiol (B) release from *P. sicula* testis during the reproductive period. VIP treatment determined a time- and dose-dependent increase of testosterone and 17β-estradiol titers compared to the controls (Table 1). These results let us to select the ideal VIP concentration (10^{-7}M) and the time exposure (120 min) for our experimental procedures.

VIP and VIP Receptor Antagonist Treatment

Winter stasis. Figure 1 depicts the effects induced by VIP and its receptor antagonists on the levels of testosterone (A) and 17β-estradiol (B) released by *P. sicula* testis during winter stasis.

Table 1. Comparison of the effects induced by administration of VIP at different concentrations and for different times on testosterone (A) and 17β-estradiol (B) titers in cultures of *Podarcis sicula* testis during the reproductive period.

Treatments	Time (min)	
A		
Control	30–60–120	70.3 ± 0.06
VIP 10^{-8}M	30	75.9 ± 0.02
VIP 10^{-7}M	30	76.3 ± 0.06
VIP 10^{-6}M	30	78.1 ± 0.05
VIP 10^{-8}M	60	86.3 ± 0.06
VIP 10^{-7}M	60	86.86 ± 0.04
VIP 10^{-6}M	60	88.91 ± 0.09
VIP 10^{-8}M	120	90.9 ± 0.05
VIP 10^{-7}M	120	93 ± 0.04
VIP 10^{-6}M	120	95.3 ± 0.05
B		
Control	30–60–120	15 ± 0.1
VIP 10^{-8}M	30	24 ± 0.1
VIP 10^{-7}M	30	25 ± 0.2
VIP 10^{-6}M	30	29 ± 0.2
VIP 10^{-8}M	60	30 ± 0.1
VIP 10^{-7}M	60	31 ± 0.1
VIP 10^{-6}M	60	32 ± 0.2
VIP 10^{-8}M	120	39 ± 0.3
VIP 10^{-7}M	120	42 ± 0.4
VIP 10^{-6}M	120	45 ± 0.2

A: Means ± SEM of testosterone levels (pg/mL) in testis control (only medium) and in testis treated with VIP at different concentrations for different times. VIP treatment determines a statistically significant ($p < 0.05$) time- and dose-dependent increase of testosterone when compared to the control. The differences between the different times and the different concentrations are statistically significant ($p < 0.05$).
B: Means ± SEM of 17β-estradiol levels (pg/mL) in testis control (medium alone) and in testis treated with VIP in different concentrations for different times. VIP treatment determines a statistically significant ($p < 0.05$) time- and dose-dependent increase of 17β-estradiol when compared to the control. The differences between the different times and the different concentrations are statistically significant ($p < 0.05$).

As regards testosterone titers, VIP, VIP/VIP1 Antagonist, and VIP/PG99-465 treatments induced a statistically significant increase compared to the time zero, control, and treatment using VIP in presence of both antagonists. Moreover, the treatment

using VIP alone produces a statistically significant increase in testosterone level compared to treatment using VIP and PG99-465. The VIP treatment in the presence of both antagonists showed no statistically significant increase compared to the control and time zero (Fig. 1A).

As regards 17β-estradiol titers, all treatments determined a statistically significant increase in hormone levels compared to the time zero and control (Fig. 1B). Furthermore, the increase of 17β-estradiol titers was comparable in all treatments, and the differences were not statistically significant when compared each other.

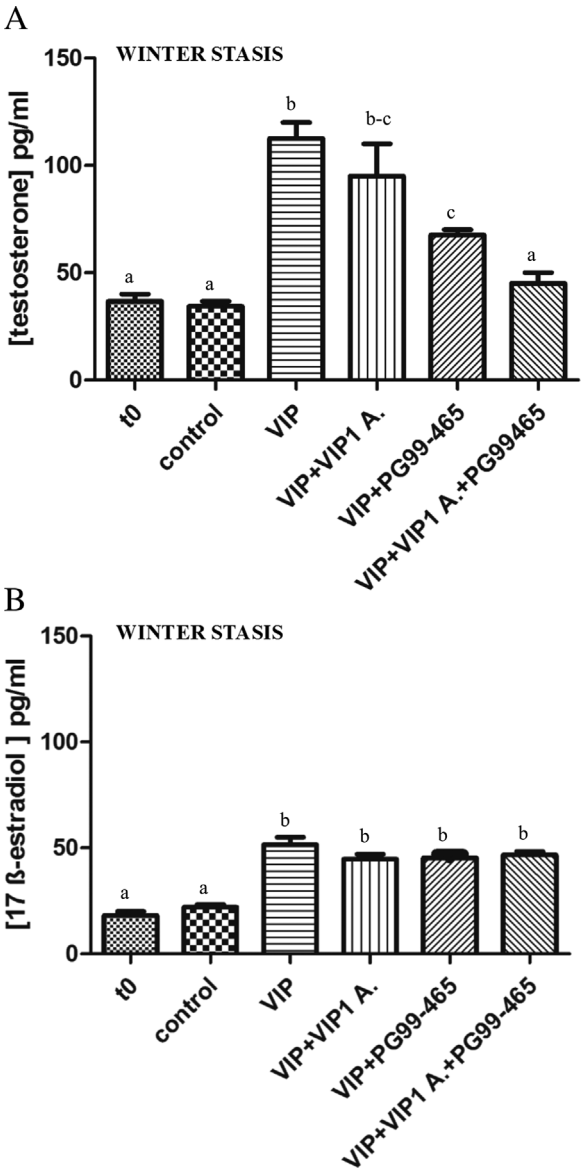

Figure 1. Winter stasis: comparison of the effects induced by administration of VIP and its receptor antagonists on testosterone (**A**) and 17β-estradiol (**B**) titers in cultures of *Podarcis* sic*ula* testis. Different letters correspond to a statistically significant difference ($p < 0.05$).

Reproductive period. Figure 2 shows the effects induced by VIP and its receptor antagonists on the levels of testosterone (A) and 17β-estradiol (B) on *P.* sic*ula* testis during the reproductive period. No statistically significant changes on testosterone levels were recorded when compared each other and to the control and time zero (Fig. 2A). Differently, VIP, VIP and VIP1 Antagonist, VIP and PG99-465 treatments showed a statistically significant increase of 17β-estradiol titers compared to the time zero and treatment with only medium (Fig. 2B). The differences among the treatments were not statistically significant. These treatments induced a statistically significant increase on 17β-estradiol titers compared to the treatment using VIP and both antagonists (Fig. 2B).

Summer stasis. Figure 3 reports the effects induced by VIP and its receptors antagonists treatments on the levels of testosterone (A) and 17β-estradiol (B) on *P.* sic*ula* testis during the summer stasis.

VIP treatment induced a statistically significant increase on testosterone levels compared to the time zero and all other treatments: all the treatments using VIP in presence of one or two antagonists determined no statistically significant changes compared to the time zero and control experiments (Fig. 3A). Furthermore, the three treatments (VIP, VIP and VIP1 Antagonist, VIP and PG99-465) induced an increase of 17β-estradiol levels comparable and statistically significant when compared to time zero, control, and treatment using VIP and both antagonists (Fig. 3A).

It is noteworthy that testosterone and 17β-estradiol levels of both time zero and control groups of reproductive period were significantly higher respect to the winter (Fig. 1) and summer (Fig. 2) stasis.

Histological Investigations

Histological investigations showed that the testis organization in culture was the same in spite of the period analyzed and the treatment used. So, we chose to report a single figure (Fig. 4A–F) depicting the tubule organization in relation to the treatment used.

Specimens treated with 10^{-7}M VIP (Fig. 4A) and with VIP and VIP1 Antagonist (Fig. 4B), VIP and the PG99-465 (Fig. 4C), and VIP in the presence of both Antagonists (Fig. 4D) showed no change in histological organization. After culture, the seminiferous tubules were characterized by the presence of all the different kind of germ cells starting from spermatogonia to spermatozoa. No change in histological organization was recorded in the control specimens (treatment 1), in the specimens from three periods (Fig. 4E) treated with medium alone, as well as

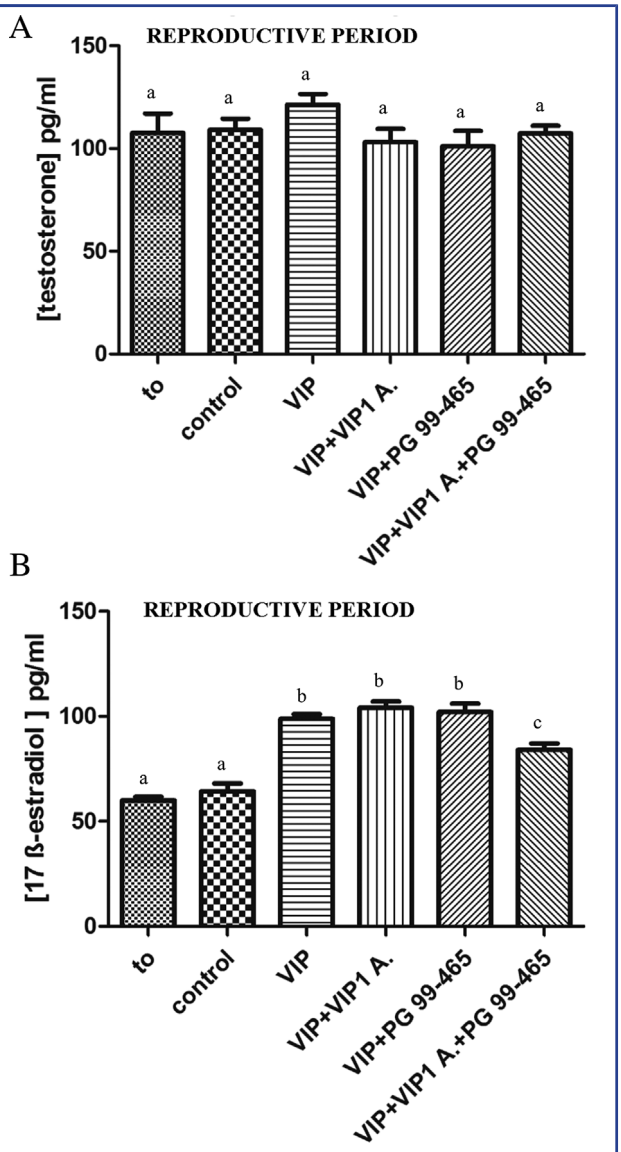

Figure 2. Reproductive period: comparison of the effects induced by treatments with VIP and its receptors antagonists on testosterone (A) and 17β-estradiol (B) titers in cultures of *Podarcis sicula* testis. Different letters correspond to a statistically significant difference ($p < 0.05$).

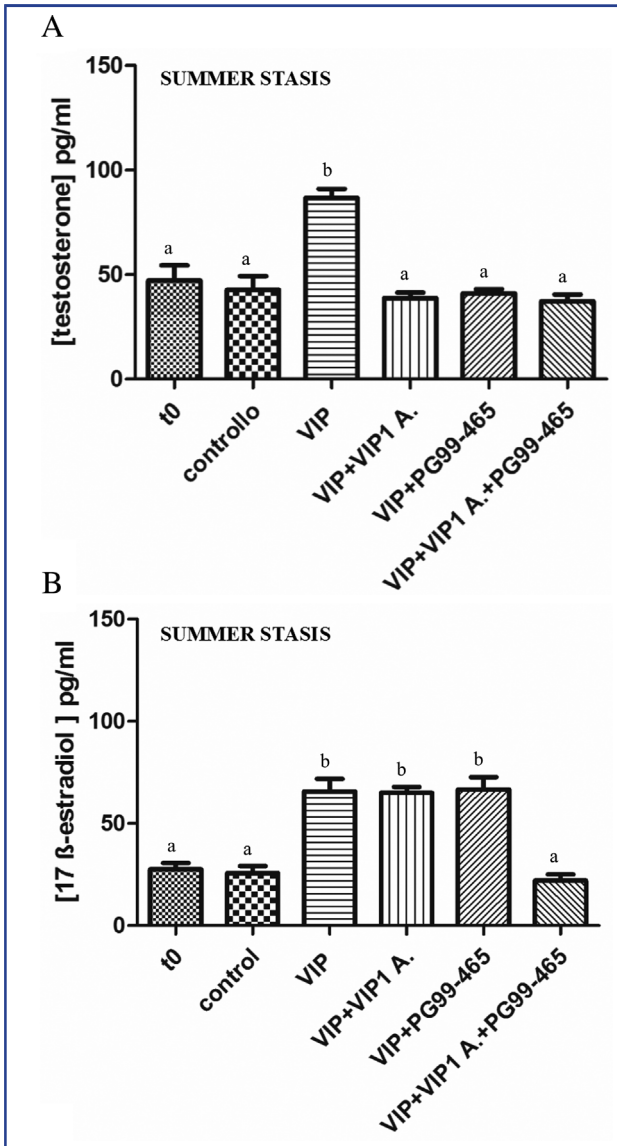

Figure 3. Summer stasis: comparison of the effects induced by the treatment with VIP and its receptors antagonists on testosterone (A) and 17β-estradiol (B) titers in cultures of *Podarcis sicula* testis. Different letters correspond to a statistically significant difference ($p < 0.05$).

in non-treated samples (Fig. 4F), including those ones of summer stasis, when only spermatogonia are present within the tubules (Fig. 4G; see also Agnese et al., 2014b).

DISCUSSION

The presence of VIP and its receptors in germ and somatic cells throughout the reproductive cycle of *P. sicula* suggested that VIP could be involved in the reproductive control of the testis activity, particularly in steroidogenesis (Agnese et al., 2014a,b). Now, by in vitro investigations carried out in significant phases of the reproductive period using VIP at different concentrations (10^{-8}M, 10^{-7}M, and 10^{-6}M) and for different times (30, 60, and 120 min), we demonstrate that in the testis, this neuropeptide is involved not only in testosterone production, as previously reported in mammals (Sherwood et al., 2000; Shioda et al., 2006; Vaudry et al., 2009), but also, for the first time, in

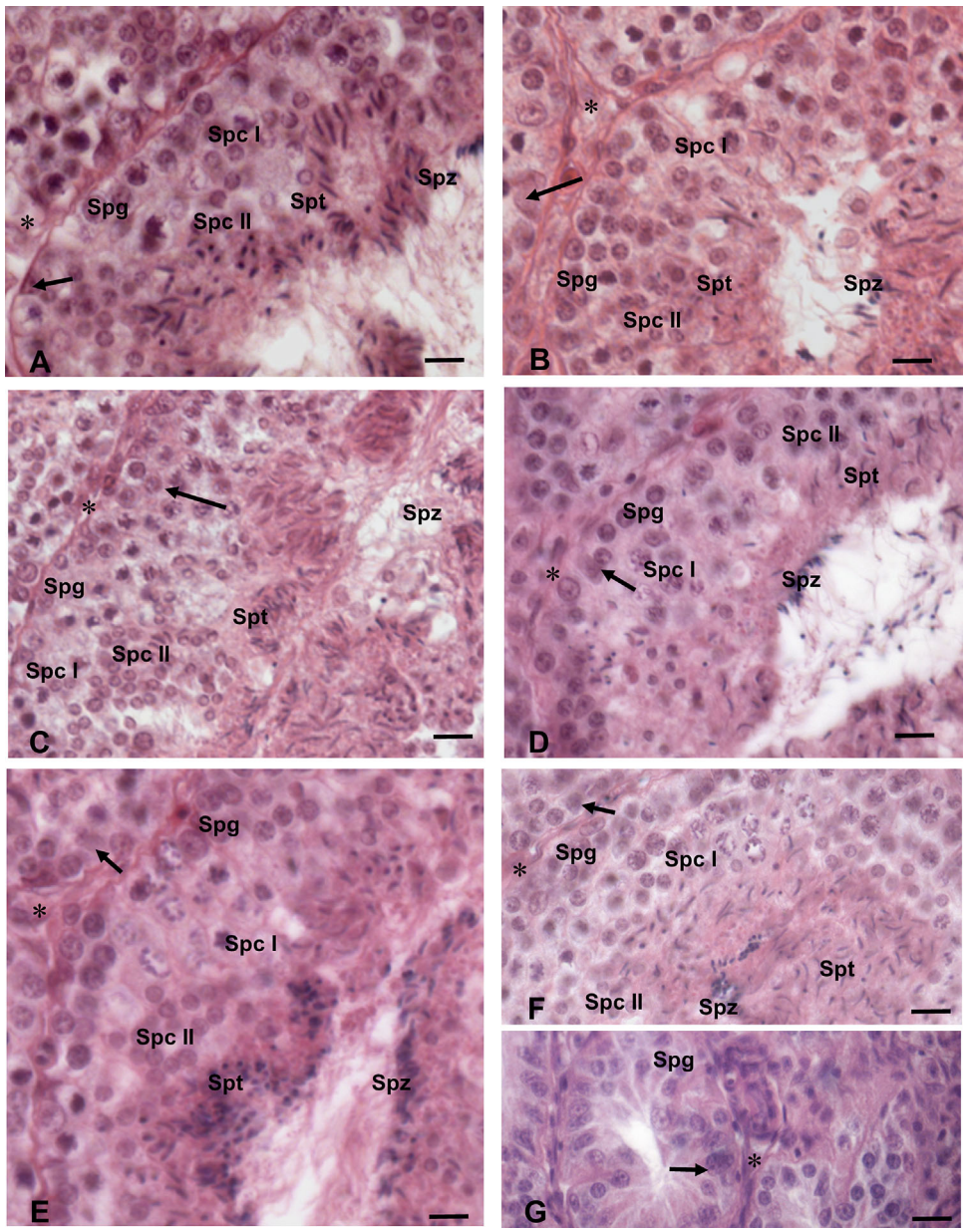

Figure 4. Histological sections of *Podarcis sicula* testes after culture stained with hematoxylin and eosin: (**A**) VIP at physiological concentration, (**B**) VIP and VIP1 antagonist, (**C**) VIP and PG99465 antagonist, (**D**) VIP and both antagonist, and (**E**) control. The tubule organization is the same in treated and control testis. Spermatogonia (SPG), primary (SPC I) and secondary (SPC II) spermatocytes, spermatids (SPT), spermatozoa (SPZ), Sertoli (arrow), and Leydig (asterisk) cells. (**F**) sections of testis not in culture. (**G**) Typical testis of summer stasis. The scale bars correspond to 20 μm.

17β-estradiol production too. Indeed, VIP induced the secretion of testosterone and 17β-estradiol in a dose- and time-dependent manner, recording the highest hormone secretion after 120 min. Such an increase recorded for a given concentration at increasing times could be due to activation of a progressively greater number of receptors as a consequence of longer exposure time to the VIP.

The hormonal evaluations have been performed in three significant periods of *Podarcis* reproductive cycle: winter stasis, reproductive period, and summer stasis, characterized by a

different hormonal profile (Angelini and Botte, '92; Raucci et al., 2005; Raucci and Di Fiore, 2009), and a different seminiferous tubules organization (Angelini and Botte, '92). We demonstrated that in the winter stasis, the treatments using VIP alone or VIP together with a single antagonist induce a statistically significant increase in testosterone and 17β-estradiol release compared to the time zero, the treatment with both antagonists, and the control too. Moreover, 17β-estradiol titers increased also in VIP treatment in presence of both antagonists. Regarding the role of VIP receptors previously, we hypothesized that VIP could regulate the production of testosterone by interacting with both $VPAC_1$ and $VPAC_2$ and that when one of the two receptors is blocked, the other one carries out the activity of the blocked receptor (Agnese et al., 2014b). Now, for the different response in the testosterone release, recorded for the two receptors during the winter stasis, we hypothesize that VIP regulates the testosterone synthesis using preferentially the $VPAC_2$ receptor; in this regard, $VPAC_1$ receptor could be used by PACAP, a neuropeptide belonging to the same family of VIP. On the other hand, as we found an increase in 17β-estradiol titers too when the VPAC receptors are blocked, we also hypothesize that differently from testosterone, the production of 17β-estradiol is under the control of another VIP-specific receptor working in addition to VPAC receptors, as reported by Zhu et al. ('95).

Differently from the winter stasis, during the reproductive period, no significant statistically increase of testosterone was recorded. This phenomenon could be due to two factors: 1) the concentration of testosterone quite high in the testis during the reproductive period (Raucci et al., 2005; Raucci and Di Fiore, 2009) and 2) the conversion of testosterone into 17β-estradiol. Indeed, as the testosterone titers is the highest level during reproductive phase, further stimulation with exogenous VIP has no effect on steroid response. Therefore, the stimulatory effect of VIP on testosterone levels could be correlated to the physiological period's concentration of this steroid hormone, typical of reproductive phase. Parallel, the invariable testosterone levels are coupled with an increase of 17β-estradiol production. The physiological mechanism underlying this last effect should involve the intervention of P450-aromatase, which is the key enzyme responsible of irreversible conversion of testosterone into 17β-estradiol (Carreau and Hess, 2010). In this regard, we can hypothesize that during reproductive period, VIP regulates the 17β-estradiol production increasing the activity of P450-aromatase, as demonstrated in hen granulosa cells, where VIP increases transcription, translation, and activity of the P450 aromatase (Johnson et al., '94). Investigations are in progress to demonstrate the presence and the activity of the aromatase in the somatic and germ cells of the *P. sicula* testis during the reproductive cycle.

Finally, we showed that during the summer stasis, experimental treatments induced a significant increase on the testosterone and 17β-estradiol release with a mechanism that involves both receptors. Concerning the testosterone increase, it is interesting to note that in this period, androgen receptors are absent (Paolucci and Di Fiore, '92), so the only presence of testosterone is insufficient to promote the spermatogenesis recall. On the other hand, the culture time is too short to induce the spermatogenesis renewal: this could explain why no modification was recorded in the organization of cultured seminiferous tubules in the summer stasis, as well as in the winter and reproductive periods, although the hormonal profile was different respect to the control and zero time.

Our considerations on the possible role of the VIP/receptors system are valid, although our data show a 17β-estradiol release during the summer substantially lower compared to quantity of hormone recorded during the reproductive phase, different from that reported in literature (Angelini and Botte, '92). The lower release of 17β-estradiol could be due to two factors: 1) The estrogen production in the testis is a process under the control of local and systemic factors. In our experimental conditions, the lack of the systemic factors could have modified the production of estrogens in the testis during the summer stasis, more significantly it compared to other phases. 2) The highest levels of estrogens in the summer stasis previously reported (Angelini and Botte, '92) refer to total trite of hormone produced both by the adrenal gland and testis.

Unfortunately, our experimental system let us to assess only the release of 17β-estradiol from the testis.

In conclusion, our data demonstrate that in the lizard *P. sicula*, the VIP/VPACR system is directly involved in male steroidogenesis, and particularly in the production of testosterone, as previously reported in mammals (Heindel et al., '92; El-Gehani et al., '98a,b,c); furthermore, as well as previously reported in the vertebrate ovary (Johnson et al., '94; Parra et al 2007; Rosas et al., 2015), VIP is involved also in the testis secretion of 17β-estradiol, this is the first evidence so far reported in vertebrate testis.

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