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Vasotocin induces final oocyte maturation and ovulation through the production of a maturation-inducing steroid in the catfish *Heteropneustes fossilis*

V. Singh, K.P. Joy *

Department of Zoology, Centre of Advanced Study, Banaras Hindu University, Varanasi 221005, India

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ABSTRACT

The study reports for the first time vasotocin (VT) induction of final oocyte maturation and ovulation through the production of the maturation-inducing steroid 17, 20β-dihydroxy-4-pregnen-3-one (MIS, 17, 20β-DP). Post-vitellogenic follicles of the catfish *Heteropneustes fossilis* were incubated with different concentrations of VT (1, 10, 100 and 1000 nM) for different time periods. Germinal vesicle breakdown [GVBD, as a marker of final oocyte maturation (FOM)] and ovulation were scored. In another series of experiments, the follicles were incubated with VT alone or in combination with VT receptor (V_1 and V_2) antagonists, and GVBD and ovulation were increased with progesterone, 17-hydroxy-4-pregnene-3, 20-dione (17-P) and 17, 20β-DP levels. VT stimulated both GVBD and ovulation in a concentration and time-dependent manner, and the responses were inhibited to varying degrees in groups incubated with the VT receptor antagonists. The V_1 antagonist inhibited the responses by 2- to 3-fold and more than the V_2 antagonist, and the combination was more potent than the separate incubation. Progestins increased time-dependently in the VT groups and the fold increase was greater for the MIS. The VT-induced steroid stimulation was significantly inhibited to near the control levels in co-incubations with both V_1 and V_2 receptor antagonists, in the order 17, 20β-DP > 17-P > P_4. The inhibition by the V_1 receptor antagonist was greater than that with the V_2 blocker, and followed the same order of inhibition described above. The results suggest that VT induces FOM and ovulation mainly through the V_1 receptors.

1. Introduction

Most vertebrate species possess at least a pair of neurohypophysial (NH) peptides, which elicit antidiurectic or reproductive activity. In teleosts, this pair is represented by vasotocin (VT) and isotocin (IT). Vasotocin is the evolutionary predecessor of vasopressin (VP) in non-mammalian vertebrates and, in addition to osmoregulation, is involved in reproductive function and behavior [2]. The occurrence of NH peptides in the vertebrate gonad has implicated a local action of the peptides in the control of gonad functions [5,31]. In mammalian species, the NH peptides have been shown to act as intra-ovarian regulators of steroidogenesis [17,36]. In lower vertebrates including fish, such studies are lacking except for a report of Rodriguez and Specker [29] who demonstrated that VT stimulated testosterone production in immature testis of rainbow trout, IT was also active but to a lesser degree. We have demonstrated for the first time the presence of VT in the catfish ovary and testis, apart from its normal distribution in the hypothalamo-hypophysial system [33]. Further, VT secretion was modulated by gonadotrophin (hCG), estradiol-17β (E_2) and progestins [34]. Reciprocally, VT modulated ovarian steroidogenesis, implying as well a regulatory role in ovarian functions [35].

The actions of the NH peptides are known to be mediated through specific G protein-coupled receptors. In mammals, at least three subtypes of receptors for VP (V_{1a}, V_2, and V_3/V_{1b}) have been characterized [21,25,38]. The V_{1a} is expressed in vascular smooth muscles and hepatocytes [25], the V_2 in the kidney [21], and the V_3/V_{1b} receptor exclusively in the pituitary [7]. Oxytocin (OT) receptor shows nearly 50% sequence homology to V_1, and 40% with V_2 receptors [9]. In fish VT receptor cDNA was first isolated in white sucker (*Catostomus commersoni*) by Mahlmann et al. [22], who demonstrated that it was functionally related with the mammalian V_1 receptor type. In euryhaline flounder (*Platichthys flesus*), Warne characterized the VT receptor [43], which showed 76% homology to the white sucker VT receptor, 62% to the mammalian V_1-type receptor, and 55% to the white sucker IT receptor. The distribution of V_1 and V_2 receptors shows species variation. Both V_1 and V_2 receptors were characterized in the ovary of pupfish (*Cyprinodon nevadensis amargosae*) [19]. Only V_2 receptor was characterized in the gonad of gray bichir (*Polypterus senegalus*) and ovary of medaka (*Oryzias latipes*) [16]. In lung fish (*Protopterus annectens*) the V_1 receptor was only characterized [15]. The IT receptor was first characterized in white sucker, and is closely related to mammalian OT receptor (66% sequence homology) and human V_{1b} receptor [13].

* Corresponding author. Fax: +91 0542 2368174.
E-mail addresses: kpjoybhu@gmail.com, keerikkattilpailyjoy@yahoo.com (K.P. Joy).

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In most teleosts including the catfish, 17, 20β-dihydroxy-4-pregnen-3-one (17, 20β-DP) is the maturation-inducing steroid (MIS), under the influence of luteinizing hormone (LH) [14,26,32]. The MIS initiates a series of phosphorylations and dephosphorylations involving participation of several protein kinases and phosphatases, to induce meiotic resumption [26,32,45], the first morphological sign of which is indicated by germinal vesicle migration and breakdown. Although the NH peptides have been implicated in eliciting spawning behavior and oviposition in fishes, reptiles and birds [10,28,30,42], their direct role in final oocyte maturation (FOM) or ovulation, as local hormones, is not known. Sirotkin et al. [37] have demonstrated VT involvement in the regulation of bovine oocyte maturation *in vitro*, which stimulated both meiosis re-initiation and completion. Since VT stimulates progestins and inhibits estrogens in post-vitellogenic follicles, and hCG (LH-like) stimulates VT secretion both *in vivo* and *in vitro* in the catfish [34,35], these hormones may form a functional cascade to regulate FOM and ovulation.

In the present work, a detailed investigation on the involvement of VT in FOM and ovulation was made using specific receptor blockers. Progestins (progesterone – P_4, 17α-hydroxyprogesterone – 17-P, the precursors, and 17, 20β-DP, the MIS) were measured and correlated with germinal vesicle breakdown (GVBD) and ovulation.

2. Materials and methods

2.1. Animal collection and acclimatization

Adult female *Heteropneustes fossilis* (50–60 g) were purchased from local fish markets in Varanasi in late pre-spawning (June, GSI – 9.8 ± 0.12%) phase of the annual reproductive cycle. The fish were acclimated in flow-through aquarium tanks under normal photoperiod and ambient temperature (13L:11D; 28 ± 2 °C) for 48 h before sampling. During the maintenance, the fish were fed daily minced goat liver *ad libitum*. The experiments were performed in accordance with local/national guidelines for experimentation in animals and all care were taken to prevent cruelty of any kind.

2.2. Chemicals

Vasotocin (Arg8 oxytocin) acetate salt (VT), 4-pregnene-3, 20-dione (progesterone, P_4), 17α-hydroxy-4-pregene-3, 20-dione (17-P), 17, 20β-dihydroxy-4-pregnen-3-one (17, 20β-DP), deamino-Pen[1], O-Me-Try[2], Arg[8] Vasopressin (V_1 receptor antagonist) and 1-adamantaneacetyl-O-Et-D-Try[2], Val[4], Abu[6], Arg[8,9] Vasopressin (V_2 receptor antagonist) were purchased from Sigma chemical company, St. Louis, MO, USA. Methanol (HPLC grade) and other chemicals (analytical grade) were purchased from E. Merck, Mumbai, India. Degassed and filtered nanopure water (Bernstead, USA) was used throughout chromatography.

2.3. Preparation of incubation medium and test compounds

The incubation medium was prepared as described earlier [32]. VT and V_1 and V_2 receptor antagonists were dissolved in HPLC grade water and stored at −20 °C. On the day of experiments just before the incubation, the stock solutions were diluted with the incubation medium to make working concentrations. For optimization of the antagonist concentration, the co-incubations were conducted with varying concentrations of V_1 and V_2 antagonists (10^{-7}, 10^{-6} and 10^{-5} M) [5]. The tested concentrations, however, did not produce any significant concentration-dependent change on the study parameters (data not shown).

2.4. Incubation of ovarian follicles with different concentrations of VT on GVBD and ovulation

The acclimatized catfish were sampled in the late pre-spawning phase (June) and ovaries were removed, weighed and transferred into a sterile petri dish containing fresh cool incubation medium. Post-vitellogenic dark green follicles (1 mm diameter) were removed with the help of sterilized fine watch maker forceps and brush. Batches of 200 follicles in triplicate from each fish ($n = 5$) were incubated with VT in different concentrations (1, 10, 100 or 1000 nM) in 5 ml incubation medium at 22 °C for 0, 8, 16 and 24 h.

2.5. Incubation of ovarian follicles with VT alone and in combination with V_1 or V_2 antagonist

Separate incubations were set up as described above for steroid assay, GVBD and ovulation studies. In the co-incubation groups, the follicles were pre-incubated with V_1 and V_2 antagonist separately or in co-incubation ($V_1 + V_2$, 10^{-6} M) for 1 h, followed by VT (100 nM) along with V_1, V_2 or $V_1 + V_2$ antagonists. Control groups (plain medium and medium containing vehicle) were set up concurrently. At every 4 h, the incubation medium was changed and collected (for steroid extraction), and replenished with fresh medium containing test compounds (except 0 h group). After completion of the incubation (0, 8, 16 and 24 h), the medium was pooled group-wise and the follicles along with the incubation medium were also collected group-wise and processed for steroid extraction and the follicles were scored for GVBD and ovulation.

2.6. GVBD scoring

After the termination of the experiments, the follicles were cleared in a clearing solution (ethanol:formalin:acetic acid; 6:3:1; [41]) and examined under a stereo-binocular for scoring the percentage of GVBD. Translucent follicles without germinal vesicle (GV) and opaque follicles containing GV were counted separately. The percentage of GVBD was determined from the total number of the follicles incubated.

2.7. Ovulation response

After the termination of the experiments, the follicles were examined under a stereo-binocular for scoring percentage ovulation. The intact follicles (unovulated) and oocytes without the follicular layer (ovulated) were counted separately. The percentage of ovulation was determined from the total number of the follicles incubated.

2.8. Steroid extraction

The tissues were homogenized separately in 4 vol of cold PBS (0.02 M, phosphate buffered saline, pH 7.4) with an ultrasonic homogenizer (XL-2000 Microson, Misonix, USA) at 0 °C for 5–10 s. The homogenate was centrifuged at 500*g* for 20 min at 4 °C and extracted with 3 vol diethyl ether, three times. The ether phase was collected and pooled, evaporated and dried under N_2 gas and stored at −20 °C till chromatography. The incubation medium was directly extracted with diethyl ether, as described above. The ether phase was collected and pooled group-wise, evaporated, dried under gas and stored at −20 °C till chromatography.

2.9. Chromatography and the validation of the assay

Chromatography and validation of the assay were described in detail earlier [34]. With the chromatographic conditions we adopted, 17-P eluted first, followed by 17, 20β-DP and P_4 with