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Hormonal action of relaxin-like gonad-stimulating substance (GSS) on starfish ovaries in growing and fully grown states

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ABSTRACT

Gonad-stimulating substance (GSS) of starfish is the only known invertebrate peptide hormone responsible for final gamete maturation, rendering it functionally analogous to gonadotropins in vertebrates. Recently, we purified GSS from the radial nerves of the starfish *Asterina pectinifera* and identified the chemical structure as a relaxin-like peptide. This study examined the hormonal action of GSS on ovaries in the growing (stage IV) and fully grown states (stage V) of the starfish. The sensitivity of oocytes to 1-methyladenine (1-MeAde) as starfish maturation-inducing hormone was enhanced as oocytes enlarged in stage V. GSS-stimulated 1-MeAde production by ovarian follicle cells was also correlated with the size of oocytes. Although 1-MeAde production was observed in whole ovaries in stage V, GSS failed to induce 1-MeAde production in young ovaries (stage IV). This suggests that follicle cells in ovaries in a growing state (stage IV) are still unresponsive to the hormonal action of GSS. According to competitive experiments using radioiodinated and radioinert GSS, however, dissociation constant (K_d) values and the number of binding sites for GSS were mostly constant in the ovaries from stages IV to V. These results strongly suggest that GSS receptors are expressed in follicle cells of ovaries in the growing state. The failure of GSS to induce 1-MeAde production in young ovaries may be due to the uncoupling of signal transduction from the receptor to 1-MeAde biosynthesis in follicle cells.

1. Introduction

Gonadotropins play important regulatory roles in reproduction in both vertebrates and invertebrates. The vertebrate gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are structurally and functionally conserved across various species, while no such molecule has been identified in invertebrates. The gonad-stimulating substance (GSS) of an echinoderm, the starfish (*Asterina pectinifera*), is the very first gonadotropin to be identified in invertebrates [1]. GSS mediates oocyte maturation in starfish by acting on the ovary to produce the maturation-inducing hormone (MIH), 1-methyladenine (1-MeAde), which, in turn, induces the maturation of oocytes [6]. In this sense, GSS is functionally identical to the vertebrate LH, especially piscine and amphibian LHs, acting on ovarian follicle cells to produce MIH to induce the final maturation or meiotic resumption of oocytes [13].

Recently, GSS was finally purified from starfish radial nerves and its chemical structure was identified [12]. This has taken approximately 50 years since it was initially discovered by Chaet and McConnaughy [1]. The purified hormone is a heterodimer composed of two different peptides, A- and B-chains with disulfide cross-linkages. On the basis of its cysteine motif, starfish GSS is classified as a member of the insulin/insulin-like growth factor (IGF)/relaxin superfamily and, more precisely, it belongs to a relaxin-like peptide family.

Furthermore, we have found that chemically synthetic GSS is able to induce 1-MeAde production in ovarian follicle cells in *A. pectinifera* [12]. Previous *in vitro* studies have shown that 1-MeAde produced under the influence of GSS is not a storage substance in follicle cells [8,14] or a breakdown product of some 1-MeAde-containing substance such as ribonucleic acids, but newly synthesized [14]. However, it is unclear when follicle cells acquire the potential for 1-MeAde production during oogenesis. To obtain more information about the hormonal action of GSS on follicle cells, this paper examined the effect of GSS on 1-MeAde production in isolated follicle cells and whole ovaries in growing and fully grown states. This study also deals with the interaction between GSS and its receptor in ovaries during the growing state.

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2. Materials and methods

2.1. Animals

Starfish, *A. pectinifera*, were collected from Yokosuka (Kanagawa, Japan), Ushimado (Okayama, Japan), Asamushi (Aomori, Japan), and Nagashima (Kagoshima, Japan). Animals were kept in circulating artificial seawater (ASW) at 15 °C and used within 2 months after collection.

2.2. Reagents

GSS was synthesized commercially (Peptide Institute Inc., Osaka, Japan). 1-MeAde and bovine serum albumin (BSA) were purchased from the Sigma Chemical Company (St. Louis, MO, USA). All other reagents were of analytical grade.

The seawater was modified Van't Hoff's ASW adjusted to pH 8.2 with 0.02 M borate buffer [5]. Calcium-free ASW (CaFSW) was prepared by replacing $CaCl_2$ in ASW with NaCl.

2.3. 1-MeAde-induced oocyte maturation

Ovarian fragments in fully grown states were isolated and washed in CaFSW, and oocytes without follicle cells were released from the fragments by transfer to ASW. Isolated oocytes were measured and placed in a small amount of test solution (usually approximately 100–200 oocytes in 0.2 ml ASW in the presence of 1-MeAde at various concentrations) and incubated at room temperature. Oocyte maturation was estimated by counting the rate of germinal vesicle breakdown (GVBD) after 1 h under a light microscope.

2.4. Isolated follicle cells

Follicle cells were separated from folliculated oocytes as described previously [7]. Briefly, the folliculated oocytes obtained from the ovary in the fully grown state were measured and treated with 1-MeAde (1 μM), and follicle cells were separated from oocytes by allowing the latter to sediment by gravity. The supernatant containing the follicle cells was collected by centrifugation at 1000*g* for 10 min at 4 °C.

Ten million follicle cells were incubated for 2 h at 20 °C in 1 ml of ASW in the presence of GSS, with occasional shaking. Then, the cell suspension was centrifuged at 1000*g* for 1 min and 1-MeAde in the supernatant was measured employing a biological assay, using authentic 1-MeAde as a standard.

2.5. Incubation of ovaries

Each segment (0.4 g wet weight) of the ovary in the growing and fully grown states, after the diameter of oocytes was measured, was incubated in ASW containing 20 nM GSS for 15 min at 20 °C in a total volume of 1 ml. After incubation, the samples were centrifuged at 1000*g* for 1 min, and the supernatant obtained was assayed for 1-MeAde.

2.6. Binding experiments

The radioiodination of GSS with $Na^{125}I$ (carrier free; GE Healthcare, UK) was carried out at room temperature according to the modified lactoperoxidase method, as described previously [16,17]. The specific activity of radioiodinated GSS was estimated at 150 μCi/μg.

The specificity of GSS binding was examined using whole homogenate of ovaries in the growing and fully grown states. These preparations were adjusted to yield a protein concentration of 10 mg/ml with the homogenizing medium (25 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4) plus 0.1% (wt/vol) BSA, and stored at −80 °C until use. The protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

The binding assay was performed in Eppendorf tubes (1.5 ml; Eppendorf Co. Ltd., Tokyo, Japan) coated with 5% (wt/vol) BSA throughout the experiments, and each preparation was assayed in duplicate. The receptor preparation (10 mg protein/ml) was diluted with the assay buffer (25 mM Tris–HCl, 10 mM $MgCl_2$, 0.1% BSA, pH 7.4). One hundred microliters of the diluted receptor preparation (25 μg protein) and 100 μl of unlabeled GSS serially diluted with the assay buffer were added to each assay tube containing 100 μl of assay buffer. Then, 100 μl of labeled GSS (approximately 100,000 cpm) diluted with assay buffer was added to each tube in a total volume of 400 μl. In total binding, 100 μl of assay buffer was added in place of the unlabeled GSS. All assay tubes were incubated for 2 h at 20 °C with shaking. At the end of the incubation, 500 μl of chilled assay buffer was added to each tube, and the tubes were centrifuged at 10,000*g* for 5 min at 4 °C. The supernatant was aspirated out and the pellet was washed twice with 1 ml of chilled assay buffer. The radioactivity of each precipitate was counted in an Aloka Auto Well Gamma System AccuFLEX γ 7010 (Aloka, Co. Ltd., Tokyo, Japan). The radioactivity in tubes containing the label and receptor preparation, but no unlabeled ligand, was designated as 100%; and the counts in other tubes were expressed as a fraction of this radioactivity. Specific binding was obtained by subtracting nonspecific from total binding. To estimate the dissociation constant (K_d) and number of binding sites (NBS), Scatchard plot analyses were performed using competition experiments with labeled and unlabeled GSS.

2.7. Microscopic observation

For histochemical observations, pieces of ovary in the growing and fully grown states were fixed in Bouin's solution. The specimens were dehydrated through a graded ethanol series and embedded in paraffin. They were sectioned at 8 μm thick. After staining with hematoxylin and eosin, the sections were observed under an Olympus BX50 light microscope equipped with an Olympus DP70 digital camera system.

3. Results

3.1. Oocyte growth in ovaries

According to Takahashi and Kanatani [15], the growth of oocytes of the starfish *A. pectinifera* can be divided into five stages on the basis of their cytological appearance (diameter of oocyte) as follows: stage I (ca. 10 μm), stage II (10–30 μm), stage III (30–70 μm), stage IV (70–150 μm), and stage V (>150 μm). Fig. 1 shows ovary cross-sections at stages III, IV, and V. At stage V, oocytes are just before or at the fully grown state. Oocytes from stages III to IV are in a growing state. Each oocyte of ovaries from stages III to V is surrounded by ellipsoidal follicle cells (Fig. 1). The follicle cells adhere firmly to each other.

3.2. 1-MeAde sensitivity in oocytes

Previous studies have shown that GSS stimulates the ovary to induce oocyte maturation by producing 1-MeAde [2,3]. An experiment was carried out to examine whether the size of oocytes was correlated with oocyte sensitivity to 1-MeAde. After the diameter of oocytes obtained from an individual ovary of *A. pectinifera* was