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Involvement of Gαs-proteins in the action of relaxin-like gonad-stimulating substance on starfish ovarian follicle cells

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ABSTRACT

Gonad-stimulating substance (GSS) in starfish is the only known invertebrate peptide hormone responsible for final gamete maturation, rendering it functionally analogous to gonadotropins in vertebrates. In breeding season (stage V), GSS stimulates oocyte maturation to induce 1-methyladenine (1-MeAde) by ovarian follicle cells. The hormonal action of GSS is mediated through the activation of its receptor, G-proteins and adenylyl cyclase. It has been reported that GSS fails to induce 1-MeAde and cyclic AMP (cAMP) production in follicle cells of ovaries during oogenesis (stage IV). This study examined the regulatory mechanism how ovarian follicle cells acquire the potential to respond to GSS by producing 1-MeAde and cAMP. Because the failure of GSS action was due to G-proteins of follicle cells, the molecular structures of Gα*s*, Gα*i*, Gα*q* and Gβ were identified in follicle cells of starfish *Asterina pectinifera*. The cDNA sequences of Gα*s*, Gα*i*, Gα*q* and Gβ consisted of ORFs encoding 379, 354, 353 and 353 amino acids. The expression levels of Gα*s* were extremely low in follicle cells at stage IV, whereas the mRNA levels increased markedly in stage V. On contrary, the mRNA levels of Gα*i* were almost constant regardless of stage IV and V. These findings strongly suggest that *de novo* synthesis of Gα*s*-proteins is contributed to the action of GSS on follicle cells to produce 1-MeAde and cAMP.

1. Introduction

In most starfish, fertilization occurs in seawater, outside the female's body. A ripe ovary contains a huge number of fully grown oocytes of almost equal size. Each oocyte still possesses a large nucleus (germinal vesicle, GV), which is arrested in late prophase of the first meiosis. The gonad-stimulating substance (GSS) of an echinoderm, the starfish, was the very first gonadotropin to be identified in invertebrates (Chaet and McConnaughy, 1959). GSS mediates oocyte maturation in starfish by acting on ovarian follicle cells to produce maturation-inducing hormone (MIH), 1-methyladenine (1-MeAde), which in turn induces the maturation of oocytes (Kanatani et al., 1969). In this sense, GSS is functionally identical to the vertebrate luteinizing hormone (LH), especially piscine and amphibian LHs, acting on ovarian follicle cells to produce MIH to induce the final maturation or meiotic resumption of oocytes (Kanatani, 1985; Nagahama et al., 1995).

It has been demonstrated that GSS purified from the radial nerves of starfish *Asterina pectinifera* is identified as a relaxin-like peptide (Mita et al., 2009). GSS binds specifically to a membrane preparation of ovarian follicles from starfish (Mita et al., 2011a). GSS also stimulates follicle cells to produce cyclic AMP (cAMP) and 1-MeAde (Mita and Nagahama, 1991; Mita et al., 2009). Thus, the action of GSS is mediated through the activation of its receptor, G-proteins, and adenylyl cyclase in follicle cells (Mita and Nagahama, 1991). However, it has been demonstrated that GSS fails to induce 1-MeAde production in follicle cells of ovaries in growing state, although GSS-stimulated 1-MeAde production by ovarian follicle cells is enhanced as increase in oocytes growth (Mita et al., 2011b, 2012). Recently, we found that the α subunit of G*s*-proteins was not detected in follicle cells during oogenesis state (Mita et al., 2012; 2013). According to Takahashi and Kanatani (1981), 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). At stage V, oocytes are just before or at the fully grown state. Oocytes at stage IV are in a growing state. Thus, it can be inferred that follicle cells in ovaries at growing state (stage IV) are not ready to receive the hormonal action of GSS, involving in its receptor, G-proteins and adenylyl cyclase.

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To obtain more information about the hormonal action of GSS on follicle cells, this study examined the signal transduction system, particularly G-proteins, for GSS in follicle cells obtained from ovaries in stages IV and V. In addition, we identified molecular structures of Gαs, Gαi, Gαq and Gβ in starfish follicle cells. The results are consistent with our earlier proposal that the potential of follicle cells to response to GSS by producing 1-MeAde and cAMP is brought by *de novo* synthesis of Gαs-proteins.

2. Material and methods

2.1. Animals

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

2.2. Reagents

GSS was synthesized commercially in Peptide Institute Inc. (Osaka, Japan). 1-MeAde, 1-methyladenosine (1-MeAdo), 4′,6-diamidino-2-phenylindole (DAPI), 3-isobutyl-1-methylxanthine (IBMX), creatine phosphate, creatine phosphokinase and guanosine-5′-triphosphate (GTP) were purchased from the Sigma Chemical Company (St. Louis, MO, USA). $Na^{125}I$ (carrier free) was obtained from GE Healthcare (Buckinghamshire, UK). 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 (Kanatani and Shirai, 1970). Calcium-free ASW (CaFSW) was prepared by replacing $CaCl_2$ in ASW with NaCl.

2.3. Microscopic observation

A folliculated oocytes of 100, 120, 140, and 160 μm in diameter isolated from ovaries of individuals were observed by a light microscope. The diameter of oocytes within ovary was an average obtained from ten oocytes. The follicle cells around each oocyte were also observed after DAPI staining as described previously (Mita, 1993). Briefly, a folliculated oocyte was stained with DAPI (0.25 mg/ml) in ASW containing 0.05% Triton X-100 for 30 min, and observed under a Laica TCS SPE-confocal laser microscope (Wetzlar, Germany).

2.4. Preparation of follicle cells

Follicle cells were prepared from folliculated oocytes as described previously (Hirai and Kanatani, 1971; Hirai et al., 1973). Briefly, both folliculated oocytes of 120–140 μm in diameter for stage IV and 150–170 μm in diameter for stage V were treated with CaFSW for 1 h at room temperature. These 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 in stages IV and V 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 quickly frozen in liquid nitrogen. Supernatants were analyzed for the amount of 1-MeAde released from follicle cells as described previously (Shirai, 1986). The frozen follicle cells were analyzed for the amount of intracellular cAMP using a BIOTRAK cAMP EIA system (GE Healthcare, Buckinghamshire, UK).

To prepare a crude membrane fraction, follicle cells in stages IV and V were homogenized using a Teflon homogenizer in 25 mM Tris–HCl (pH 7.4) containing 10 mM $MgCl_2$, and the homogenate was centrifuged at 10,000*g* for 30 min at 4 °C. The precipitate was washed twice with the same homogenizing medium and used as the crude membrane fraction. The protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Binding experiments

The radioiodination of GSS with $Na^{125}I$ (carrier free) was carried out at room temperature in accordance with the modified lactoperoxidase method, as described previously (Mita et al., 2011a). The specific activity of radioiodinated GSS was estimated at 150 μCi/μg. The specificity of GSS binding was examined using whole homogenate of isolated follicle cells in stages IV and V. These preparations were adjusted to yield a protein concentration of 10 mg/ml with the homogenizing medium plus 0.1% (wt/vol) BSA, and stored at −80 °C until use.

The binding assay was performed as described previously (Mita et al., 2007, 2011a). To estimate the dissociation constant (K_d) and number of binding sites (NBS), Scatchard plot analyses (Scatchard, 1949) were performed using competition experiments with labeled and unlabeled GSS.

2.6. Adenylyl cyclase assay

A modified version of a method described previously (Mita and Nagahama, 1991) was employed. Briefly, the adenylyl cyclase reaction was carried out for 20 min at 20 °C by adding the crude membrane fraction of follicle cells in stages IV and V to medium containing 40 mM Tris–HCl (pH 7.8), 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma Chemical Co., St. Louis, MO, USA), 10 mM creatine phosphate, 0.03 mg/ml creatine phosphokinase, 1 mM ATP, 6 mM $MgCl_2$, and 20 mM NaN_3 in a total volume of 0.1 ml. The reaction was stopped by adding 0.1 ml of 0.1 M EDTA and boiling for 3 min. Concentrations of cAMP were determined using a BIOTRAK cAMP EIA system (GE Healthcare, Buckinghamshire, UK).

2.7. Immunoblotting

The crude membrane fraction (5 μg protein) was dissolved in gel sample buffer and boiled for 5 min. Aliquots were loaded into the lanes of a 10/20% sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) mini slab (10/20% gel) (Cosmobio, Tokyo, Japan) and resolved by electrophoresis, as described previously (Mita et al., 2012). Proteins separated by SDS–PAGE were transferred to an Immobilon membrane (Millipore, Billerica, MA, USA) by electroblotting, as described previously (Mita et al., 2012). The membrane was rinsed in Tris-buffered saline (TBS) consisting of 20 mM Tris–HCl (pH 7.5) and 150 mM NaCl, blocked with 5% non-fat dry milk in TBS containing 0.1% Tween 20 (TTBS), and incubated with a 1:1000 dilution of anti-Gαs, anti-Gαi, or anti-Gβ antibodies (Merck, Darmstadt, Germany) in TTBS overnight at 4 °C. After three washes with TTBS, the membrane was incubated with a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Cosmobio, Tokyo, Japan). After three further washes with TTBS, phosphatase activity was visualized by treating the membrane with 0.2 mM 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and nitroblue tetrazolium in 100 mM diethanolamine buffer (pH 9.5) containing 5 mM $MgCl_2$.

2.8. cDNA cloning

Total RNA was extracted from the follicle cells in stage V of *A. pectinifera* after homogenization with Sepasol (Nacalai tesque,