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Contribution of *de novo* synthesis of Gα*s*-proteins to 1-methyladenine production in starfish ovarian follicle cells stimulated by relaxin-like gonad-stimulating substance

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ABSTRACT

In starfish, the peptide hormone gonad-stimulating substance (GSS) secreted from nervous tissue stimulates oocyte maturation to induce 1-methyladenine (1-MeAde) production by ovarian follicle cells. The hormonal action of GSS on follicle cells involves its receptor, G-proteins and adenylyl cyclase. However, GSS failed to induce 1-MeAde and cAMP production in follicle cells of ovaries during oogenesis. At the maturation stage, follicle cells acquired the potential to respond to GSS by producing 1-MeAde and cAMP. Adenylyl cyclase activity in follicle cells of fully grown stage ovaries was also stimulated by GSS in the presence of GTP. These activations depended on the size of oocytes in ovaries. The α subunit of *Gs*-proteins was not detected immunologically in follicle cells of oogenesis stage ovaries, although Gα*i* and Gα*q* were detectable. Using specific primers for Gα*s* and Gα*i*, expression levels of Gα*s* in follicle cells were found to increase significantly as the size of oocytes in ovaries increased, whereas the mRNA levels of Gα*i* were almost constant regardless of oocyte size. These findings strongly suggest the potential of follicle cells to respond to GSS by producing 1-MeAde and cAMP is brought by *de novo* synthesis of Gα*s*-proteins.

1. Introduction

The gonad-stimulating substance (GSS) of an echinoderm, the starfish, was the very first gonadotropin to be identified in invertebrates. GSS mediates oocyte maturation in starfish by acting on ovarian follicle cells to produce maturation-inducing hormone, 1-methyladenine (1-MeAde), which in turn induces the maturation of oocytes [1,2]. Recently, GSS was purified from the radial nerves of starfish *Asterina pectinifera*, and it was identified as a relaxin-like peptide [3]. It has also been demonstrated that GSS binds specifically to a membrane preparation of ovarian follicles from starfish [4,5] and that isolated follicle cells cultured with GSS showed a dose-related increase in cyclic AMP (cAMP) production, coinciding with an increase in 1-MeAde production [3,6]. Thus, the action of GSS is mediated through the activation of its receptor, G-proteins, and adenylyl cyclase in follicle cells [6]. However, GSS fails to induce 1-MeAde production in follicle cells of ovaries in growing states [7,8]. According to Takahashi and Kanatani [9], the growth of oocytes of *A. pectinifera* can be divided into five stages on the basis of their cytological appearance (diameter of the 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 in a growing state (stage IV) are not ready to receive the hormonal action of GSS, involving in its receptor, G-proteins and adenylyl cyclase.

To elucidate the regulatory mechanism of acquisition of potential by follicle cells to respond to GSS action up to the breeding season, this study examined the signal transduction system of GSS in follicle cells of ovaries in growing and fully grown states.

2. Materials and methods

2.1. Materials

GSS was synthesized commercially (Peptide Institute Inc., Japan). 1-MeAde, GTP and GTP-γS were purchased from Sigma (USA). Anti Gα*s*, Gα*i*, and Gα*q* antibodies were obtained from Merck (Germany). All other reagents were of analytical grade.

The seawater was modified Van't Hoff's artificial seawater (ASW) adjusted to pH 8.2 with 0.02 M borate buffer [10]. Calcium-free ASW (CaFSW) was prepared by replacing $CaCl_2$ in ASW with NaCl.

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2.2. Preparation of follicle cells

Starfish, *A. pectinifera*, were collected from Yokosuka (Kanagawa, Japan), Choshi (Chiba, Japan), Ushimado (Okayama, Japan), Asamushi (Aomori, Japan), and Omura (Nagasaki, Japan). Follicle cells were separated from folliculated oocytes as described previously [11].

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 quickly frozen in liquid nitrogen. Supernatants were analyzed for the amount of 1-MeAde released from follicle cells as described previously [12]. The frozen follicle cells were analyzed for the amount of intracellular cAMP using a BIOTRAK cAMP EIA system (GE Healthcare, UK).

To prepare a crude membrane fraction, follicle cells 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, USA).

2.3. Adenylyl cyclase assay

A modified version of a method described previously [6] was employed. Briefly, the adenylyl cyclase reaction was carried out for 20 min at 20 °C by adding the crude membrane fraction to medium containing 40 mM Tris–HCl (pH 7.8), 1 mM 3-isobutyl-1-methylxanthine (IBMX), 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, UK).

2.4. Immunoblotting

The crude membrane fraction (30 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, Japan) and resolved by electrophoresis, as described previously [8]. Proteins separated by SDS–PAGE were transferred to an Immobilon membrane (Millipore, USA) by electro-blotting, as described previously [8]. 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-Gαq antibodies (Merck, 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, 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 $MgCl_2$.

2.5. cDNA cloning

Total RNA was extracted from the follicle cells of *A. pectinifera* after homogenization with Sepasol (Nacalai Tesque, Japan) as an RNA extraction solution. A poly(A)$^+$ RNA fraction was obtained using Oligotex-dT30 (Nippon Gene, Japan). First-strand cDNA was synthesized by using a SMARTer RACE cDNA Amplification Kit (Clontech, USA) in accordance with the manufacturer's instructions. Oligonucleotide primers for the cDNA cloning of Gαs, Gαi or Gαq were designed in accordance with sequences (*GenBank: AY534105* [Gαs in *Strongylocentrotus purpuratus*], *GenBank: AY534106* [Gαs in *Lytechinus variegatus*], *GenBank: X66378* [Gαi in *A. pectinifera*], *GenBank: AY534107* [Gαq in *L. variegates*], *GenBank: AY534108* [Gαq in *S. purpuratus*, long form], *GenBank: AY534109* [Gαq in *S. purpuratus*, short form]). The 5′- and 3′-RACE products encoding Gαs, Gαi or Gαq were amplified with these primers to determine the open reading frames (ORF) of cDNAs for Gαs, Gαi or Gαq.

All PCR products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide. The agarose gel slices containing the PCR-product band were excised under UV illumination, and DNA was purified from the agarose plug using a QIAquick® Gel Extraction kit (Qiagen, USA), followed by an ethanol precipitation. Amplified products were cloned into pGEM-T® easy vector in the pGEM-T® easy system (Promega, USA). The DNA sequence data were determined on ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, USA) using a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA).

2.6. Real-time quantitative PCR analysis

Real-time quantitative PCR was performed using a StepOnePlus system (Applied Biosystems) to examine changes in gene expressions of Gαs and Gαi. Total RNA was isolated from follicle cells, and treated with DNase (Invitrogen, USA). First strand cDNA was synthesized from 1 μg total RNA using the M-MLV Reverse Transcriptase (Promega) with an oligo(dT)-anchor primer. Primer pairs used for real-time quantitative PCR analyses were as follows: Gαs-F: 5′-CGATGTAGGAGGGCAGAGAG-3′, Gαs-R: 5′-TAACTACTGCACGCGACCAC-3′; Gαi-F: 5′-GGAGGACAGCGTTCAGAGAG-3′, Gαi-R: 5′-ATGCGGTTCATTTCCTCATC-3′; and β-actin-F: 5′-TCACAGAGCGTGGCTACTCTTTC-3′, β-actin-R: 5′-TGATGTCACGCACGATTTCA-3′.

β-Actin (*GenBank: AB298788*) was used as the internal standard. The reaction mixture contained SYBR Green Real-Time PCR Mix (Toyobo, Japan), 400 nM each of forward and reverse primers, and 300 ng of cDNA in a final volume of 20 μl. PCR was run with a standard cycling program: 95 °C for 3 min, 40 cycles of 95 °C, 15 s; 60 °C, 15 s; 72 °C, 15 s. An external standard curve was generated by serial 10-fold dilution of cDNA obtained from the follicle cell, which had been purified and its concentration measured. To confirm the specificity of the amplification, the PCR products were subjected to melting curve analysis and gel electrophoresis. Results were analyzed using StepOnePlus 2.0 software (Applied Biosystems, USA) and expressed as relative mRNA expression per β-actin.

3. Results and discussion

3.1. GSS-induced 1-MeAde production in follicle cells

It has been reported that GSS fails to stimulate 1-MeAde production in follicle cells obtained from ovaries in a growing state [7,8]; therefore, an experiment was carried out to confirm this finding and to examine when follicle cells acquire the potential for GSS-stimulated 1-MeAde production during oogenesis. When follicle cells isolated from folliculated oocytes up to 140 μm in diameter in stage IV were incubated for 2 h with GSS at 20 nM, 1-MeAde (Fig. 1A) and cAMP production (Fig. 1B) were not observed. GSS could induce 1-MeAde and cAMP production in follicle cells obtained from folliculated oocytes more than 140 μm in diameter. The amount of 1-MeAde produced in the media increased significantly as oocyte size increased (Fig. 1A). GSS also stimulated an increase in intracellular levels of cAMP after oocyte