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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

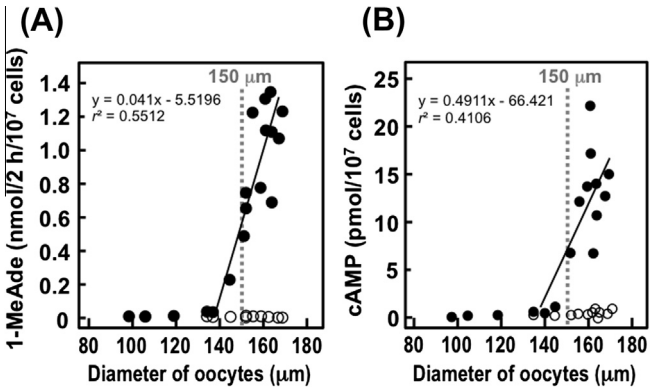

Fig. 1. Effect of GSS on 1-MeAde (A) and cAMP production (B) by follicle cells prepared from ovaries in growing (stage IV) and fully grown stages (stage V) of the starfish *A. pectinifera*. Follicle cells prepared from each folliculated oocyte in diameter as indicated were incubated with ASW in the absence (○) and presence of 20 nM GSS (●) for 2 h at 20 °C. The diameter of oocytes within each ovary was an average obtained from ten oocytes. The amount of 1-MeAde released into the medium was estimated using a biological assay with an analytical curve generated with authentic 1-MeAde. Intracellular cAMP content was determined by EIA. The dotted lines at 150 μm show the cut-off between stages IV and V. Regression lines and correlation coefficients were: 1-MeAde production, $y = 0.041x - 5.5196$, $r^2 = 0.5512$; cAMP production, $y = 0.4911x - 66.421$, $r^2 = 0.4106$.

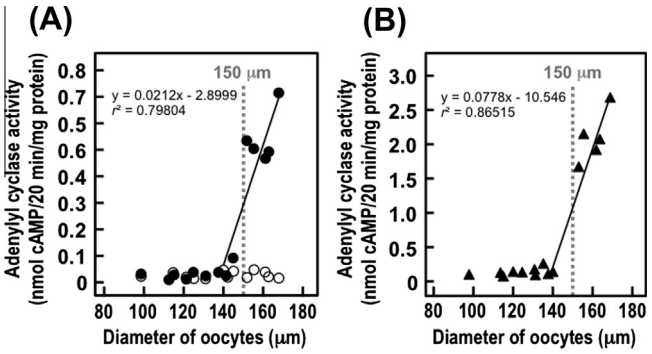

Fig. 2. Effect of GSS (A) and GTP-γS (B) on adenylyl cyclase activity in follicle cells prepared from ovaries in growing (stage IV) and fully grown stages (stage V) in the starfish *A. pectinifera*. Follicle cells were prepared from each folliculated oocyte of the diameter indicated. The diameter of oocytes within each ovary was an average obtained from ten oocytes. The adenylyl cyclase activity was assessed for 20 min at 20 °C by adding the crude membrane fraction of follicle cells to medium containing 40 mM Tris–HCl (pH 7.8), 0.1 mM IBMX, 10 mM creatine phosphate, 0.03 mg/ml creatine phosphokinase, 1 mM ATP, 6 mM $MgCl_2$, 20 mM NaN_3 and either 20 nM GSS in the absence (○) or presence (●) of 0.1 mM GTP or 0.1 mM GTP-γS (▲). The dotted lines at 150 μm show the cut-off between stages IV and V. Regression lines and correlation coefficients were: GSS + GTP, $y = 0.0212x - 2.8999$, $r^2 = 0.79804$; GTP-γS, $y = 0.0778x - 10.546$, $r^2 = 0.86515$.

growth (Fig. 1B). This suggests that the production of cAMP as well as 1-MeAde is strongly related to the size of oocytes during oogenesis, particularly when close to the mature stage.

In previous studies [8], GSS receptors have been observed in follicle cells during oogenesis, although the K_d and number of binding sites in follicle cells at stage IV was inferior to those at stage V. This strongly suggests that GSS receptors are present in follicle cells of ovaries in the growing state in starfish. However, it is contradictory that GSS exerts its hormonal action on follicle cells to produce 1-MeAde caused by an increase in cAMP level just before and in the fully grown state.

3.2. Effect of GSS on adenylyl cyclase activity

Because adenylyl cyclase directly produces cAMP, the next experiment was carried out to examine the effect of GSS on adenylyl cyclase activity in follicle cells during oogenesis. Crude membranes of follicle cells were prepared from folliculated oocytes from 98 to 170 μm in diameter. Activities of adenylyl cyclase in the membrane preparations were measured in the presence of 20 nM GSS. Without GTP, GSS had no effect on adenylyl cyclase activity. Adding 0.1 mM GTP, GSS increased adenylyl cyclase activity in parallel with an increase in the size of oocytes more than 140 μm in diameter (Fig. 2A). This suggests that G-proteins play an important role in adenylyl cyclase activation.

Previous studies have shown that a nonhydrolyzable GTP analog, GTPγS, stimulates adenylyl cyclase activity in follicle cells of just before and fully grown stages [8]. However, neither GSS nor GTPγS could stimulate adenylyl cyclase activity in follicle cells obtained from folliculated oocytes less than 140 μm in diameter (Fig. 2A and B). These findings suggest that signal transduction associated with GSS in follicle cells of ovaries in oogenesis is unable to couple with 1-MeAde biosynthesis. Presumably, the hormonal signal of GSS is not passed to G-proteins in these immature follicle cells.

3.3. Identification of Gα-subunits in follicle cells

G*αs* and G*αi* have been shown to interact directly with upstream signaling components for adenylyl cyclase [13], and the molecular structures of G*αs* and G*αi* were identified in ovarian follicle cells. cDNA fragments were obtained from mRNAs from ovarian follicle cells in a fully grown state by RT-PCR. cDNA sequences encoding G*αs* and G*αi* were determined using a SMART RACE cDNA Amplification Kit (Clontech, USA). The cDNAs for G*αs* (1140 bp) and G*αi* (1065 bp) consisted of ORFs encoding 379 and 354 amino acids, respectively. We also identified the cDNA sequence of G*αq*. The ORF for G*αq* consisted of 1062 bp and encoded 353 amino acids. These sequences were submitted to the GenBank (*GenBank: AB856985* [G*αs*], *GenBank: AB856986* [G*αi*], *GenBank: AB856987* [G*αq*]). The cDNA and amino acid sequences of G*αi* in follicle cells were in accordance with those in oocytes reported by Chiba et al. [14]. The homologies of these Gα proteins in starfish were about 70% compared with those in mammals.

3.4. Western-blotting of Gα-subunits in follicle cells

Previous studies have shown that G*αs* is undetectable in follicle cells during oogenesis [8]. To confirm this finding and to further determine whether G*αs*, G*αi* and G*αq* are present in follicle cells during oogenesis, immunoblotting experiments using antibodies against G*αs*, G*αi* and G*αq* were performed in follicle cells obtained from folliculated oocytes of 100, 120, 140, and 160 μm in diameter. Recognition sites of antigens for commercial anti-G*αs*, anti-G*αi* and anti-G*αq* antibodies were observed in the amino acid sequences of G*αs*, G*αi* and G*αq* proteins in starfish follicle cells (data not shown).

G*αs* with a molecular weight of 45 kDa was recognized by the antibody in follicle cells from oocytes of 160 μm in diameter (Fig. 3). However, the band for G*αs* was faint in samples from oocytes of 140 μm in diameter. These bands were hardly detected in follicle cells from oocytes of 100 and 120 μm in diameter. On the other hand, 41 kDa G*αi* and 41 kDa G*αq* were present in follicle cells regardless of oocyte size (Fig. 3). These results strongly suggest that G*αs* proteins are absent in follicle cells of ovaries during oogenesis.

3.5. Expression of Gαs and Gαi proteins

To confirm whether G*αs* is absent in follicle cells during oogenesis, the mRNA levels of G*αs* in follicle cells in growing states were compared with those in fully grown oocytes. The expression levels

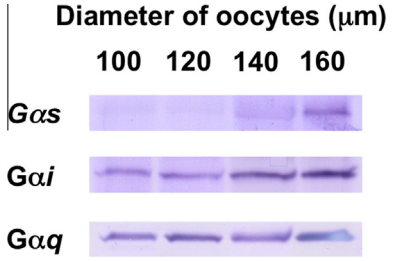

Fig. 3. Immunoblotting after SDS–PAGE of crude membrane preparations of ovarian follicle cells with anti-G*αs*, anti-G*αi*, and anti-G*αq* antibodies. Follicle cells were prepared from folliculated oocytes of 100, 120, 140, and 160 μm in diameter. The diameter of oocytes within each ovary was an average obtained from ten oocytes. The crude membrane fraction (5 μg protein/well) of follicle cells was separated by SDS–PAGE and used for Western blotting, as described in the Section 2.

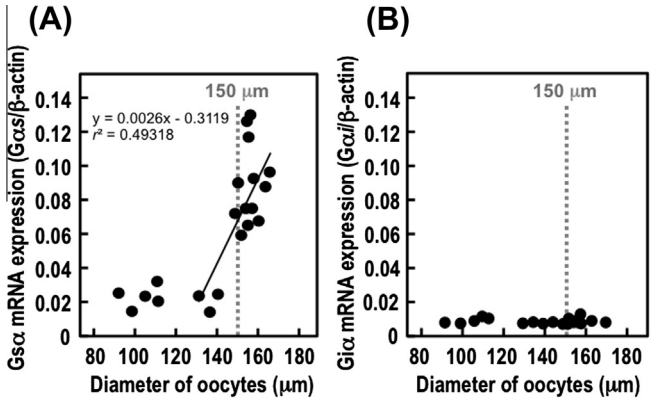

Fig. 4. Expression levels of G*αs* (A) and G*αi* (B) mRNAs in follicle cells prepared from ovaries in growing (stage IV) and fully grown stages (stage V) in the starfish *A. pectinifera*. Follicle cells were prepared from folliculated oocytes of the diameter indicated. The diameter of oocytes within each ovary was an average obtained from ten oocytes. The expression levels of G*αs* and G*αi* mRNAs were measured by real time-PCR using specific primers for G*αs* and G*αi*, respectively, as described in the Section 2. The dotted lines at 150 μm show the cut-off between stages IV and V. The diameter of oocytes within each ovary was an average obtained from ten oocytes. Regression line and correlation coefficient were: $y = 0.0026x - 0.3119$, $r^2 = 0.49318$.

of G*αs* measured using real-time PCR with specific primers were extremely low in follicle cells of oocytes up to 140 μm in diameter (Fig. 4A). The mRNA levels increased significantly as oocyte sizes increased above 140 μm in diameter (Fig. 4A). These results strongly suggest that G*αs* proteins in follicle cells are newly synthesized in follicle cells during the maturation stage. It is thus considered that the failure of GSS to stimulate follicle cells in the growing state to produce 1-MeAde is because of a lack of G*αs*.

In contrast, the mRNA levels of G*αi* were low and remained almost constant during oogenesis (Fig. 4B). Because G*αi*-proteins were present in follicle cells of growing-stage folliculated oocytes (Fig. 3), it may not be necessary to synthesize them newly.

It was confirmed from the present study that the effect of GSS on 1-MeAde production by starfish follicle cells is initiated by the receptor-mediated activation of G*αs* proteins, resulting in the activation of adenylyl cyclase and the formation of cAMP. The most important finding in this study is that G*αs* proteins were newly synthesized in follicle cells until maturation stage. The data suggest that *de novo* synthesis of G*αs* proteins contributes to the response to GSS in follicle cells resulting in the production of 1-MeAde. This is new evidence as a regulatory mechanism of hormonal action by G-proteins. Further studies on a first trigger for *de novo* synthesis of G*αs* proteins should provide useful insights into the regulation of GSS-stimulated 1-MeAde production by starfish ovarian follicle cells.

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