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# Inhibitory Effect of a SALMFamide Neuropeptide on Secretion of Gonad-Stimulating Substance from Radial Nerves in the Starfish *Asterina pectinifera*

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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 SALMFamide family is also known to an echinoderm neuropeptide. The present study examined effect of SALMFamide 1 (S1) on oocyte maturation of starfish *Asterina pectinifera*. Unlike GSS, S1 did not induce spawning in starfish ovary. In contrast, S1 was found to inhibit GSS secretion from radial nerves by treatment with high $K^+$ concentration. Fifty percent inhibition was obtained by 0.1 mM S1. S1 did not have any effect on GSS- and 1-MeAde-induced oocyte maturation. Following incubation with a S1 antibody and subsequently with rhodamine-conjugated second antibody, neural networks were observed in ovaries. The networks were restricted mainly to their surface with little evidence of immunoreactivity inside the basement membranes. This indicates that neural networks are distributed in the ovarian wall. The result further suggests that S1 plays a role in oocyte maturation to regulate GSS secretion from the nervous system.

**Key words:** oocyte maturation, gonad-stimulating substance, SALMFamide, neural network, starfish

## INTRODUCTION

In most starfish, oocytes in a ripe ovary remain arrested at the end of the first prophase stage of meiosis. Such immature oocytes can not be fertilized and as in vertebrates, starfish oocyte maturation and ovulation are induced by hormonal substances. Chaet and McConnaughy (1959) first reported that a water extract of *Asterias* radial nerves can induce the shedding of gametes when injected into the coelomic cavity of ripe animals. The active substance contained in the nerve extract is considered to be a peptide hormone named gonad-stimulating substance (GSS) (Kanatani 1967; Kanatani and Shirai, 1969). GSS is the primary mediator of oocyte maturation in starfish. However, the affect of GSS on oocyte maturation is indirect. Resumption of meiosis in immature oocytes and release from the ovary are induced by a second mediator identified as 1-methyladenine (1-MeAde) (Kanatani *et al*., 1969; Kanatani, 1969, 1973, 1985). Thus, GSS induces 1-MeAde production in ovarian follicle cells (Kanatani *et al*., 1969; Hirai and Kanatani, 1971; Hirai *et al*., 1973; Kanatani, 1973; Shirai, 1986) by way of activation of its receptor, G-protein and adenylyl cyclase (Mita and Nagahama, 1987; Mita *et al*., 1989; Mita and Nagahama, 1991).

The hormonal action of 1-MeAde together with its receptor localized on the oocyte plasma membrane (Kanatani and Shirai, 1970; Yoshikuni *et al*., 1988; Tadenuma *et al*., 1992) is to transfer the signal to the cytoplasm where the maturation (or M phase)-promoting factor (MPF) is activated (Kishimoto and Kanatani, 1976). MPF is the direct trigger of germinal vesicle breakdown (GVBD) (Kishimoto and Kanatani, 1976) and follicular envelop breakdown (Kishimoto *et al*., 1984). MPF was first discovered in maturing frog oocytes (Masui and Markert, 1971) and has since been identified as a complex of Cdc2 kinase with cyclin B (Gautier *et al*., 1988, 1990).

The SALMFamide family is characteristic echinoderm

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neuropeptides (Elphick *et al.*, 1991). SALMFamide 1 (S1) was originally isolated from *Asterias rubens* as an octapeptide with the amino acid sequence Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-$NH_2$ (Elphick *et al.*, 1991). S1 is distributed ubiquitously in starfish nervous system (Elphick *et al.*, 1991; Newman *et al.*, 1995a, b). While there is increasing information on pharmacological effects of S1 on neuromuscular organs such as tube feet and apical muscle in starfish (Elphick *et al.*, 1995), there is no data available on the possible affects of S1 on oocyte maturation. Since both S1 and GSS are peptides present in the starfish nervous system, we have examined influence of S1 on oocyte maturation in starfish *Asterina pectinifera*. The organization of neural networks in *A. pectinifera* ovaries was also examined by using an antibody for the S1.

## MATERIALS AND METHODS

### Animals

Starfish, *Asterina pectinifera*, were collected at Yokosuka (Kanagawa, Japan), Asamushi (Aomori, Japan) and Nagashima (Kagoshima, Japan). Animals were kept in circulating artificial seawater ('My sea', Jamarin Laboratories, Osaka, Japan) at 15°C and were used within two months after collection.

### Reagents

1-MeAde and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Lot. No. U70K9173) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The S1 peptide was synthesized by NIBB Center for Analytical Instruments (Okazaki, Japan). The peptide antiserum was raised in rabbit and produced as described previously (Moore and Thorndyke, 1993). Artificial seawater (ASW) comprised 433 mM NaCl, 22 mM KCl, 11 mM $CaCl_2$, 24 mM $MgCl_2$, 23 mM $MgSO_4$, and 50 mM borate buffer, pH 7.3 (Takahashi, 1985). The composition was essentially the same as that of artificial plasma of the starfish as described previously (Shirai *et al.*, 1982). To prepare the high concentration of $K^+$ seawater (KSW), this was adjusted to 100 mM, with KCl substituted for NaCl. GSS was prepared from the lyophilized radial nerves of *A. pectinifera*, as described previously (Kanatani *et al.*, 1976; Shirai, 1986).

### GSS secretion

Release of GSS from radial nerves was induced by treatment with high potassium seawater as described previously (Takahashi, 1985). Intact radial nerve cords were isolated from arms of *A. pectinifera* with forceps. The nerves collected were placed in ASW. To induce GSS secretion, isolated radial nerve cords (10 mg wet weight) were incubated in KSW for 20 min at 20°C in a total volume of 1.0 ml and then transferred into ASW for 20 min at 20°C. The procedure was repeated using the same solution. After fifth treatment with KSW, the test solution was centrifuged at 1,500×g for 5 min at room temperature and the supernatant used for GSS assay. The affect of S1 on KSW-stimulated GSS secretion was tested by co-incubation of radial nerve cords with both KSW and a range of S1 concentrations.

### Biological assay of GSS

Each fragment (7–10 mg wet weight) of ovaries was incubated with ASW containing GSS in a total volume of 0.2 ml. After 1hr incubation at 20°C, the samples were examined to determine whether or not spawning had occurred. The concentration of GSS was determined by the method described previously (Shirai, 1986), using authentic GSS as a standard reference. Amounts of GSS were expressed as the original nerve weight (dry nerve weight equivalent). Concentrations of GSS required for 50% spawning were also determined as *Ka*.

### 1-MeAde-induced oocyte maturation

Isolated oocytes without follicle cells were prepared as described previously (Shirai, 1986). These oocytes were placed in a small amount of test solution (usually about 200-300 oocytes in 0.2 ml ASW) and incubated at 20°C. Oocyte maturation was estimated by counting the rate of GVBD after 1 hr under a light microscope.

### Immunocytochemistry

Ovaries of *A. pectinifera* were fixed in a fresh solution of 4% paraformaldehyde in ASW for 5 hr at 4°C. After fixation the speci-

**Table 1.** Effect of GSS and SALMFamide 1 on starfish ovaries

| Conditions | Experiment number | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Control | – | – | – |
| GSS (0.025 mg/ml) | – | + | – |
| (0.05 mg/ml) | ++ | +++ | +++ |
| (0.1 mg/ml) | +++ | +++ | +++ |
| SALMFamide 1 (0.05 mM) | – | – | – |
| (0.1 mM) | – | – | – |
| (0.2 mM) | – | – | – |

Ovarian fragment (7–10mg) was incubated with ASW in the presence of GSS or S1 at indicated concentrations in a total volume of 0.2 ml. After 1 hr incubation at 20°C, the samples were examined to determine whether or not spawning had occurred. (+++): spawning occurred and most of oocytes were matured, (++): about 50% oocytes were matured, (+): a few oocytes were matured, and (–): no spawning occurred.

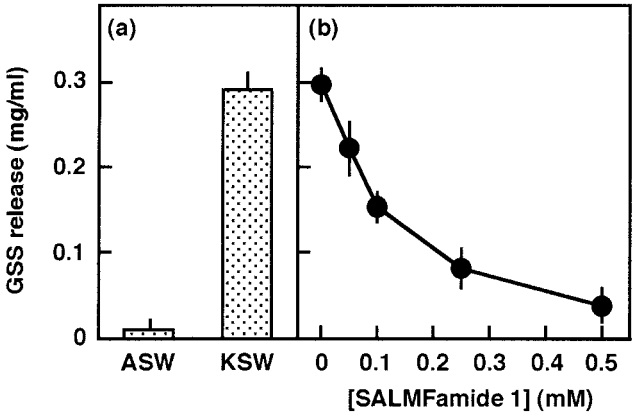


**Fig. 1.** Effect of SALMFamide 1 on release of GSS from isolated radial nerve cords by the treatment of high potassium seawater. The isolated radial nerve cords (10 mg/ml) were incubated in 100 mM $K^+$ seawater (KSW) for 20 min at 20°C in the absence (a) and presence of S1 at the indicated concentrations (b), and then transferred into normal ASW for 20 min. The procedures were repeated using the same solution. After fifth treatment with KSW, the amount of GSS released was assayed. In (a), ASW: the radial nerves were treated with ASW alone. Each value is the mean of four separate experiments. Vertical bars show SE.

mens were washed with phosphate buffered saline (PBS) and pre-incubated with PBS-Triton X-100 (0.1%) containing 2% bovine serum albumin for 1 hr at 4°C. The ovaries were incubated with antiserum of S1-antibody for 12 hr at 4°C. The preparations were rinsed several times with PBS and subsequently incubated with TRITC-conjugated goat anti-rabbit IgG for 2 hr at 4°C. After rinsing with PBS, dehydration in an ethanol series and clearing with benzoylbenzoic acid/benzyl alcohol (1 : 1), the specimens were observed with a LCM510 confocal laser microscope (Carl Zeiss, Germany) using a 568 nm laser for excitation and a 585 nm LP filter for emission. For observation with a light microscope, thin sections were cut with a microtome and stained with hematoxylin.

## RESULTS

Previous studies have shown that spawning occurs under the influence of GSS (Chaet and McConnaughy, 1959; Kanatani, 1969; Mita *et al.*, 1994). Since the neuropeptide S1 is also present in starfish nervous system (Elphick *et al.*, 1991; Newman *et al.*, 1995a, b), we examined the possibility that also S1 stimulates spawning. Although more than 0.05 mg/ml of GSS induced spawning of incubated after 1 hr in accordance with our previous observations (Mita and Nakamura, 1994), spawning was not induced following incubation of ovaries with S1 at concentrations of 0.05 mM, 0.1 mM, and 0.2 mM (Table 1).

**Table 2.** Effect of SALMFamide 1 on GSS-induced spawning in starfish ovaries

| Conditions | *Ka* (mg/ml) |
|---|---|
| Control | 0.036 ± 0.007 |
| SALMFamide 1 (0.05 mM) | 0.038 ± 0.006 |
| (0.1 mM) | 0.038 ± 0.007 |
| (0.2 mM) | 0.036 ± 0.006 |

*Ka* stands for a concentration of GSS required for 50% spawnning. Each value is the mean ± SE obtained in three separate experiments.

It has been shown that GSS can be secreted from radial nerves following treatment with high potassium seawater (Takahashi, 1985). The present study was carried out to confirm this finding and to further determine whether S1 influences GSS secretion. After isolated radial nerve cords

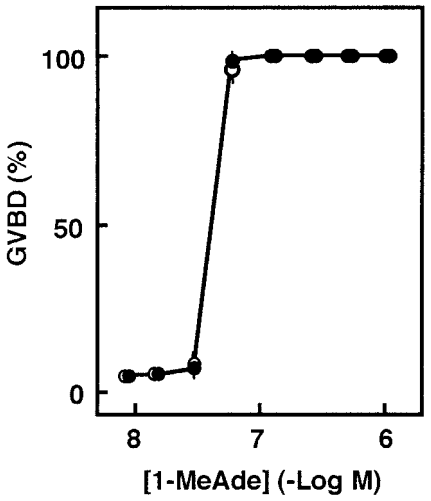


**Fig. 2.** Effect of SALMFamide 1 on 1-MeAde-induced oocyte maturation in starfish. Isolated oocytes were incubated in 1-MeAde as indicated concentrations under the absence (○) and presence of S1 (0.1 mM) (●). Oocyte maturation was estimated by counting the rate of GVBD after 1hr under a light microscope. Each value is the mean of three separate experiments. Vertical bars show SE.

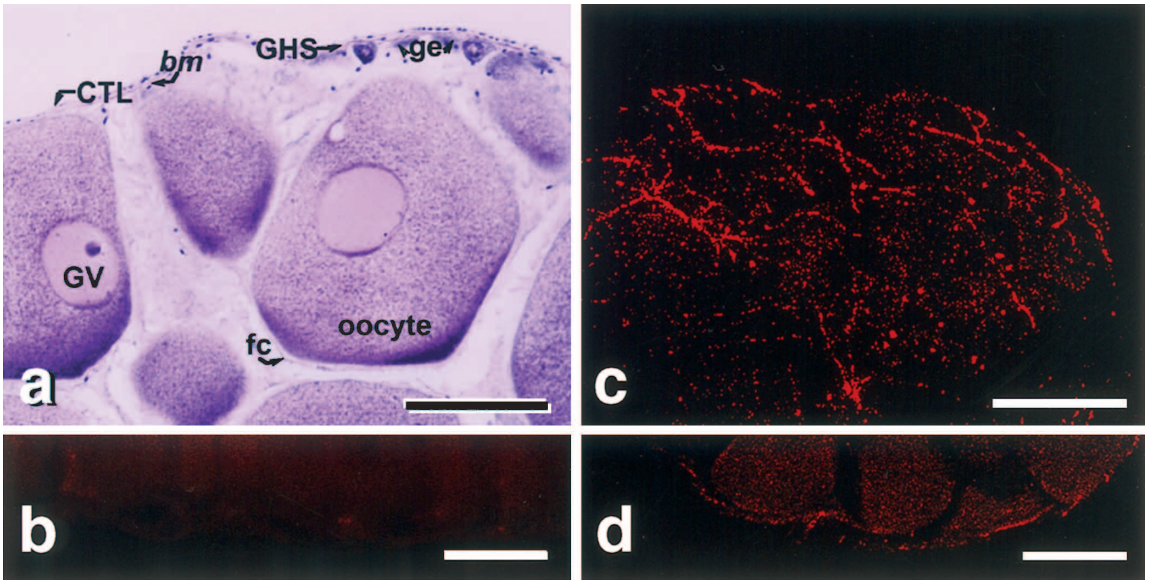


**Fig. 3.** The distribution of SALMFamide 1 in starfish ovary. The immunoreactivity was observed in overlay (c) and section (d) by a conforcal LASER scanning microscope following incubation with anti-SALMFamide 1 primary antibody and subsequently with rhodamine-conjugated second antibody. In a control experiment (b) the specimen was incubated without anti-S1 antibody. In (a), the thin section of ovary was stained with hematoxylin and observed by a light microscope. Bars show 100 μm. Abbreviations: bm, basement membrane; CTL, connective tissue layer; fc, follicle cells; ge, germinal epithelium; GHS, genital haemal sinus; GV, germinal vesicle.

of *A. pectinifera* were treated with KSW and ASW repeatedly, GSS secreted was observed in the media (Fig. 1a). ASW alone did not stimulate GSS release from radial nerves. When co-incubated with the neuropeptide S1, GSS secretion from radial nerves was significantly inhibited (Fig. 1b). Fifty percent inhibition was obtained with 0.1 mM S1.

In contrast, S1 did not influence GSS-induced spawning. About 0.036 mg GSS equivalent to dry nerve weight per 1 ml induced 50% spawning (*Ka*)regardless of the presence of S1 at concentrations of 0.05, 0.1 and 0.2 mM (Table 2).

Previous studies have shown that 1-MeAde produced by ovarian follicle cells under the influence of GSS induces oocyte maturation (Kanatani *et al*., 1969; Kanatani, 1973, 1985). 1-MeAde induced oocyte maturation with a dose-dependent manner. However, S1 did not have any effect on 1-MeAde-induced oocyte maturation (Fig. 2).

Examination of *A. pectinifera* ovaries, employing an antibody for S1 indicated extensive neural networks over the ovarian surface (Fig. 3c). The networks were stricken mainly to the connective tissue layer and little evidence of immunoreactivity was seen inside their basement membrane such as germinal epithelium (Fig. 3a, d). In a control experiment without S1 antibody, there was a faint fluorescence in ovaries (Fig. 3b). Similar networks were observed in ovaries stained with DiI, a fluorescent neural tracer (data not shown).

## DISCUSSION

The present study showed that the neuropeptide S1 inhibited KSW-induced GSS secretion from radial nerves of *A. pectinifera*. Although it is uncertain whether S1 can inhibit natural GSS secretion during the breeding season, there is no information about the mechanism of natural GSS secretion from nervous tissues. Thus, it is an important finding in this study that GSS secretion is inhibited by S1. However, it has been reported that S1 is failed to detect in the coelomic fluid in starfish *A. rubens* (Elphick *et al*., 1995). It might be unexpected that S1 plays an inhibitory effect on GSS secretion from nervous system in coelomic fluid. On the other hand, high concentrations of S1 have been found to be present in the radial nerve cords (Elphick *et al*., 1995). It is possible therefore that GSS secretion is inhibited by S1 within nervous system.

This study also showed that, unlike GSS, S1 does not induce spawning. In addition, there was no effect of S1 on GSS-induced spawning and 1-MeAde-induced oocyte maturation. This suggests that S1 does not have a direct effect on oocyte maturation in starfish. Rather, the affect is indirect with S1 having an inhibitory role in the regulation of GSS levels. Once GSS is released from S1 inhibition and secreted from nervous tissue, GSS would stimulate 1-MeAde production in ovarian follicle cells and then 1-MeAde would induce oocyte maturation *via* MPF activation.

In this study, S1-immunoreactive neural networks were also observed in starfish ovaries. The networks are restricted mainly to ovarian wall. This suggests that the nervous system is also an important part of ovarian structure and it is possible that not only S1 but also GSS is present in the ovarian nervous system. Thus, it may be that GSS secreted from the ovarian nervous system directly stimulates oocyte maturation. Further work will test the idea that GSS is present in the ovarian neural network. The present study also suggests that there is the strong possibility that neuropeptide S1 plays a role in oocyte maturation by the local regulation of GSS secretion. It also remains for further investigations to test the idea that S1 and GSS regulation of oocyte maturation arises from either radial nerve cords sources, ovarian sources (or both). However, our data clearly support the idea that S1 is responsible for inhibiting GSS release during the oogenesis that proceeds to the breeding season. Further investigations of the role of S1 in the regulation of GSS secretion during the breeding season should provide useful insights into the hormonal control of meiotic maturation of starfish oocytes.

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