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Role of Ecto-ATP Diphosphohydrolase in Ovarian Follicle Cells of the Starfish *Asterina pectinifera*

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ABSTRACT—A high activity of ecto-ATP diphosphohydrolase (ATPDase, EC 3.6.1.5) is present on the surface of ovarian follicle cells of the starfish *Asterina pectinifera*. It is known that follicle cells play an important role in oocyte maturation by producing 1-methyladenine (1-MeAde) as the maturation-inducing substance. This study was undertaken to determine if ecto-ATPDase is associated with 1-MeAde production. 1-MeAde production is induced by a gonad-stimulating substance (GSS) secreted from nervous tissue. It has also been shown that G-proteins and adenylyl cyclase are involved in the action of GSS in 1-MeAde production. When isolated follicle cells were incubated with the ecto-ATPDase metabolites, adenosine and AMP, a slight decrease in intracellular cyclic AMP level was observed. Adenosine also inhibited adenylyl cyclase in the presence of GTP. Pertussis toxin reversed adenosine-induced inhibition, suggesting that adenosine acts via the Gi receptor. On the other hand, GSS could induce 1-MeAde production and an increase in cyclic AMP level regardless of the absence or presence of AMP or adenosine. GSS-dependent 1-MeAde production markedly decreased after washing of follicle cells with seawater containing low concentrations of Ca^{2+}, but the activity of ecto-ATPDase remained constant. Neither NaF nor adenylimidodiphosphate, which are inhibitors of ecto-ATPDase, had any effect on GSS-induced 1-MeAde production. Thus, it is unlikely that ecto-ATPDase is directly involved in 1-MeAde production in starfish ovarian follicle cells. Since an increase in cyclic AMP level is indispensable for 1-MeAde production, it may be possible that adenosine and AMP produced by ecto-ATPDase play a role in prevention of precocity before oocyte maturation stage.

INTRODUCTION

In most starfish, the fully-grown ovary is filled with immature oocytes, each surrounded by an envelope consisting of follicle cells. The follicle cells adhere firmly to each other and to the ovary wall (Kanatani and Shirai, 1969). Resumption of meiosis in these oocytes and release from the ovary are induced by 1-methyladenine (1-MeAde) (Kanatani *et al.*, 1969; Kanatani, 1969, 1973), which is produced by follicle cells after stimulation with a gonad-stimulating substance (GSS) secreted from nervous tissue (Chaet and McConnaughy, 1959; Kanatani *et al.*, 1969; Hirai and Kanatani, 1971; Kanatani, 1973; Shirai, 1986).

It has been shown that upon incubation of follicle cells with GSS, there is a dose-related increase in cyclic AMP production, coincident with an increase in 1-MeAde production (Mita *et al.*, 1987, 1989; Mita and Nagahama, 1991). It has also been reported that G-proteins and adenylyl cyclase are involved in the action of GSS on 1-MeAde production (Mita and Nagahama, 1991). Thus, the action of GSS on 1-MeAde production by follicle cells is initiated by receptor-mediated activation of Gs, resulting in the activation of adenylyl cyclase and formation of cyclic AMP.

Recently, we reported that a Mg^{2+}-dependent ATP-diphosphohydrolase (ATPDase, EC 3.6.1.5) was present in starfish ovarian follicle cells (Mita *et al.*, 1998, 2000). ATPDase belongs to the group of enzymes that hydrolyze ATP. However, there are substantial differences between ATPDase and ATPase (Plesner, 1995). The ATPDase in starfish ovarian follicle cells, which has an optimum pH between 6.0 and 7.5, can hydrolyze various triphosphonucleosides (ATP, GTP, CTP, UTP and ITP) and diphosphonucleosides (ADP, GDP, CDP, UDP and IDP), but not monophosphonucleosides (Mita *et al.*, 1998, 2000). ATPDase activity is insensitive to ATPase inhibitors, such as ouabain, vanadate and oligomycin, but it is strongly inhibited by sodium fluoride and adenylimidodiphosphate (AppNHp) (Mita *et al.*, 1998). In contrast to its biochemical properties, little is known about the physiological roles of ATPDase in starfish ovarian follicle cells. Since starfish follicle cells play an important role in oocyte maturation by producing 1-MeAde, this study was undertaken to determine if ATPDase is associated with the process of 1-MeAde production.

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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

ATP, ADP, AMP, adenosine and adenylimidodiphosphate (AppNHp) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2'- (or -3'-)- *O*-(Trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP) was obtained from Molecular Probes (Eugene, OR, USA). Pertussis toxin (PTX) from Seikagaku Kogyo (Tokyo, Japan) was activated by incubation with 40 mM dithiothreitol (DTT) for 30 min at 30°C before use. All other reagents were of analytical grade. The seawater used was modified van't Hoff's artificial seawater (ASW) adjusted to pH 8.2 with 20 mM borate buffer (Kanatani and Shirai, 1970). To prepare seawater with low concentrations of Ca^{2+}, NaCl was substituted for $CaCl_2$. The GSS was prepared from the lyophilized radial nerves of *A. pectinifera*, as described previously (Kanatani *et al.*, 1976; Shirai, 1986). Amounts of GSS in the present report refer to the original nerve weight (dry nerve weight equivalent).

Preparation of follicle cells

Follicle cells were isolated from folliculated oocytes as described previously (Mita, 1985; Mita *et al.*, 1988). Briefly, folliculated oocytes were squeezed out of the ovaries and washed with ASW. The folliculated oocytes were treated with 1-MeAde (1 μM) and, after the oocytes had been allowed to settle by gravitation, the supernatant containing the follicle cells was collected and filtered through nylon mesh (40 μm) to remove any contaminating oocytes. The filtrate was centrifuged at 1,000 × *g* for 5 min at 4°C, and the sedimented follicle cells were washed twice with ASW.

The follicle cells were homogenized with 50 mM Tris-HCl buffer at pH 7.5, and the homogenate was used for assays of ATPDase, lactate dehydrogenase (Vassault, 1983), 1-methyladenosine (1-MeAdo) ribohydrolase (Mita, 1986) and adenylyl cyclase (Mita and Nagahama, 1991). Protein was measured by the method of Lowry *et al.* (1951). For preparation of crude membrane fractions, the follicle cells were homogenized with 40 mM Tris-HCl containing 6 mM $MgCl_2$ at pH 7.8. The homogenate was centrifuged at 15,000 × *g* for 30 min at 4°C. The sediment was washed twice with the same buffer. The precipitate was used as the crude membrane fraction.

Ecto-ATP diphosphohydrolase assay

The standard reaction mixture for the ATPDase assay contained 2 mM $MgCl_2$, 25 mM Tris-HCl buffer, pH 7.5, and either 2 mM ATP or 2 mM ADP in a total volume of 0.1 ml. After incubation for 10 min at 20°C, 0.1 ml of 10% trichloroacetic acid (TCA) was added to the reaction mixture. The amount of Pi liberated from ATP and ADP as the result of the enzymatic reaction was measured according to the method of Fiske and SubbaRow (1925).

Ecto-ATPDase activity was also measured in intact cells. The follicle cells were suspended in 1.0 ml of ASW in the presence of either ATP (2 mM) or ADP (2 mM). After incubation for 10 min at 20°C, the cell suspension was centrifuged at 1,000 × *g* for 1 min and the supernatant assayed for Pi as described above.

Adenylyl cyclase assay

The reaction mixture for the adenylyl cyclase assay contained 1 mM ATP, 6 mM $MgCl_2$, 20 mM NaN_3, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 10 mM creatine phosphate, 0.03 mg creatine kinase, 1 mM DTT and 40 mM Tris-HCl at pH 7.8. Crude membranes of follicle cells (0.05 mg protein) were incubated in the reaction mixture (0.1 ml) for 20 min at 20°C. The reaction was stopped by adding 0.1 ml of 0.1 M EDTA and boiling for 3 min. Cyclic AMP was measured with the BIOTRAK cyclic AMP enzyme immunoassay system (Amersham Pharmacia Biotech, Buckinghamshire, England). All measurements were done in duplicate.

Incubation of follicle cells

Ten million follicle cells were incubated for 2 hr at 20°C in 1.0 ml of ASW with occasional shaking. After incubation, the cell suspension was centrifuged at 1,000 × *g* for 1 min and the supernatant assayed for 1-MeAde. The concentration of 1-MeAde was determined by the method described previously (Shirai, 1974, 1986) using authentic 1-MeAde as a standard reference. The sedimented cells were quickly frozen in liquid N_2 and used for determination of cyclic AMP. The cyclic AMP was measured as described above, following extraction of the cells with 5% TCA.

Observation of fluorescence

A folliculated oocyte was stained with TNP-ATP, a fluorescent ATP analogue, at a concentration of 0.1 mM in ASW, and observed with a confocal laser microscope (Axioplan 2 Universal Microscope, Carl Zeiss, Germany) based on a 488 nm laser for excitation and a 505 nm LP filter for emission.

RESULTS

When a homogenate obtained from follicle cells was incubated with either ATP or ADP at a concentration of 2 mM at 20°C, ATP and ADP were rapidly catalyzed to AMP and Pi (Table 1). About 40,000 or 28,000 nmoles of Pi were formed from ATP or ADP, respectively, by 1 mg of whole homogenate protein per 10 min. This is due to ATPDase activity, as reported previously (Mita *et al.*, 1998). In contrast to ATPDase, the specific activities of lactate dehydrogenase for glycolysis, 1-MeAdo ribohydrolase for 1-MeAde production and adenylyl cyclase for signal transduction were about 56, 12 and 0.1 nmoles produced/10 min/mg of homogenate protein, respectively (Table 1). Comparison of these specific activities indicates that high ATPDase activity is present in starfish ova-

Table 1. Specific activities of ATPDase, lactate dehydrogenase, 1-methyladenosine ribohydrolase and adenylyl cyclase in starfish follicle cell homogenate

Enzyme	Specific activity (nmol produced/10 min/mg protein)
ATPDase (ATP)	40,000±2,000
ATPDase (ADP)	28,000±2,000
Lactate dehydrogenase	56±2
1-Methyladenosine ribohydrolase	12±1
Adenylyl cyclase	0.10±0.03

Values are means±SEM of three separate experiments.

rian follicle cells.

ATPDase activity was also observed by incubating folliculated oocytes with ASW in the presence of either ATP (2 mM) or ADP (2 mM) (Table 2). However, ATPDase activity was relatively lower in defolliculated oocytes. This strongly suggests that the ATPDase is located in follicle cells. TNP-ATP was used as a fluorescent analogue of ATP to examine the location of ATPDase. Since TNP-ATP is only fluorescent when bound to protein (Hiratsuka, 1982), the location of ecto-ATPDase would be observed as fluorescence. Folliculated oocytes were stained with TNP-ATP at a concentration of 0.1 mM, and fluorescence was observed with a confocal laser microscope; a 488 nm laser was used for excitation and an LP 505 nm filter was used for emission to cut off wavelength less than 505 nm. As shown in Fig. 1, intense fluorescence was observed in the follicle envelope surrounding immature oocytes (Fig. 1b). In a control experiment without TNP-ATP, there was a faint fluorescence in folliculated oocytes (Fig. 1d). These suggest that ATPDase is located on the follicle cell surface. Furthermore, when intact follicle cells were incubated in ASW containing ATP, ADP or AMP at a concentration of 2 mM, Pi released was related into the medium in the presence of either ATP or ADP (Fig. 2). The hydrolysis of extracellular ATP and ADP was dependent on the number of follicle cells. In contrast, incubation of cells with AMP did not induce Pi release. These results strongly suggest that the catalytic moiety of ATPDase is located outside the cell as an ecto-ATPDase.

Table 2. Ecto-ATPDase activity in starfish folliculated and defolliculated oocytes

Sample	Oocyte Number (10^3 oocytes/ml)	ATPDase activity (μmol Pi released/ml)	
		ATP (2 mM)	ADP (2 mM)
Folliculated oocytes	5	0.09±0.01	0.06±0.01
	10	0.14±0.08	0.09±0.01
	20	0.23±0.05	0.16±0.01
Defolliculated oocytes	5	0.02±0.01	0.01±0.01
	10	0.03±0.01	0.02±0.01
	20	0.05±0.01	0.04±0.01

Folliculated oocytes were directly prepared by squeezing starfish ovaries using forceps. Defolliculated oocytes were obtaned by spawning after treatment of ovaries with Ca^{2+}-free seawater. Value are means±SEM of three separate experiments.

Since starfish ovarian follicle cells play an important role in oocyte maturation by producing 1-MeAde, an experiment was performed to examine the relationship between ecto-ATPDase activity and 1-MeAde production. In general, extracellular adenine nucleotides and adenosine are agonists coupled with the adenylyl cyclase inhibitory system. When follicle cells were incubated with ASW in the presence of adenosine or AMP at a concentration of 2 mM, the level of cyclic AMP was significantly lowered as compared with the control (Table 3). In crude membrane fractions, adenosine

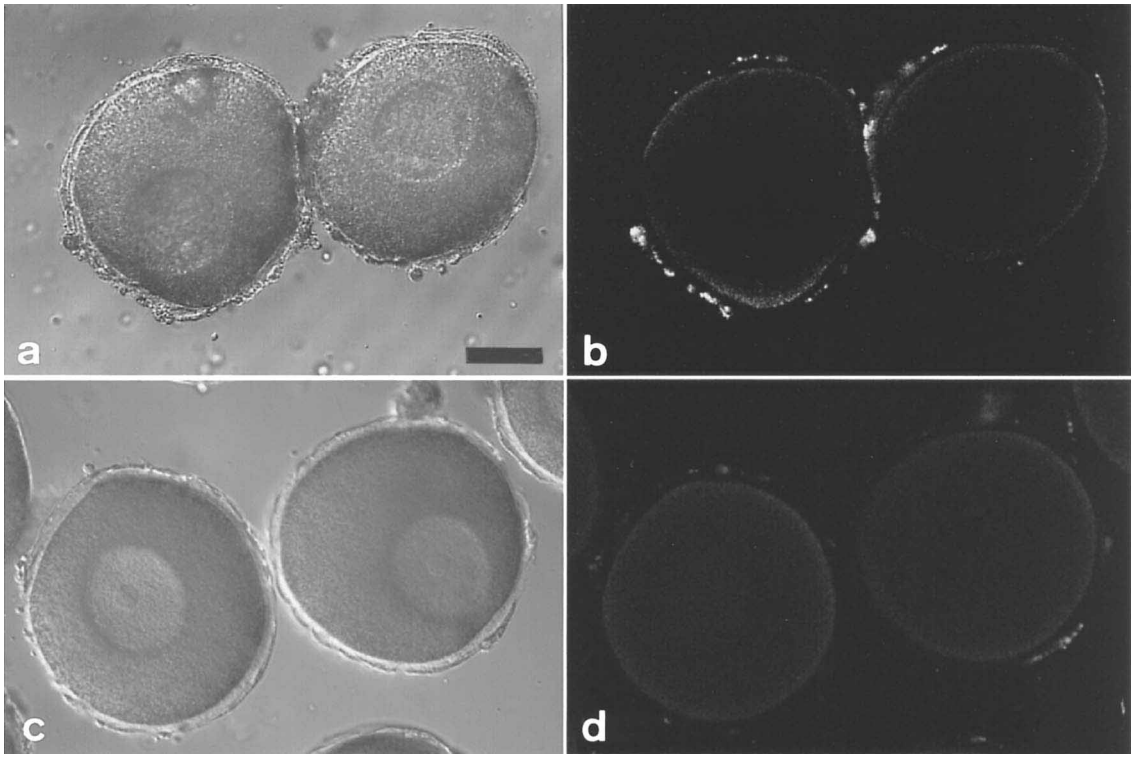

Fig. 1. Micrographs of folliculated oocytes treated with TNP-ATP. Folliculated oocytes were suspended in ASW in the absence (c and d) and presence of TNP-ATP (0.1 mM) (a and b). The preparation was observed by light (a and c) and fluorescence excited by a 488 nm laser (b and d) with confocal laser microscopy. Bar is 50 μm.

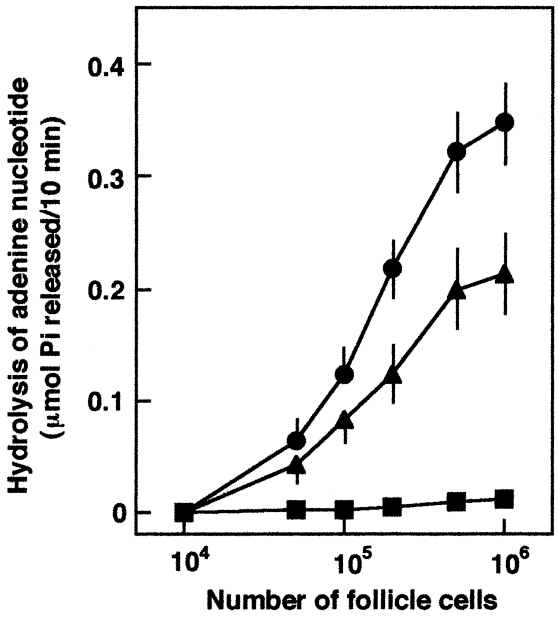

Fig. 2. Hydrolysis of extracellular ATP, ADP and AMP by intact starfish follicle cells. Intact follicle cells at the numbers indicated were incubated for 10 min at 20°C with ASW in the presence of 2 mM ATP (●), ADP (▲) or AMP (■) in a total volume of 0.1 ml. After incubation, the cell suspension was centrifuged at 1,000×*g* for 1 min and the supernatant was used for Pi assay. Each value is the mean of three separate experiments. Vertical bars show SEM.

induced a concentration-dependent inhibition of adenylyl cyclase activity (Fig. 3a). GTP at a concentration of 0.1 mM enhanced this action, with half maximal inhibition at 0.4 mM adenosine. Furthermore, PTX reversed the adenosine-induced inhibition of adenylyl cyclase in a dose-dependent manner (Fig. 3b), although PTX did not have reversal effect on adenylyl

Table 3. Effect of adenosine on 1-MeAde production and cyclic AMP concentration in starfish follicle cells

Conditions	1-MeAde production (nmol/2h/10^7 cells)	cyclic AMP concentration (pmol/10^7 cells)
Zero time control	–	2.0 ±0.1
Control	ND	1.9 ±0.1
Adenosine (2 mM)	ND	1.5 ±0.1[a]
AMP (2 mM)	ND	1.4 ±0.1[a]
GSS (0.1 mg/ml)	0.89±0.06	7.6 ±0.5
GSS+adenosine (2 mM)	0.88±0.05	7.3 ±0.3[b]
GSS+AMP (2 mM)	0.89±0.05	7.3 ±0.3[b]

Values are means±SEM of three separate experiments. ND, not detectable.
[a] Significantly lower than the control value (P<0.1).
[b] Not significant as compared with the value for GSS alone (P>0.1).

cyclase activity under the absence of adenosine. These results strongly suggest that ATPDase metabolites such as AMP and adenosine inhibit adenylyl cyclase activity via a signal transduction system coupled with a Gi-protein. However, increases in cyclic AMP level and 1-MeAde production were induced by GSS (0.1 mg/ml) regardless of the absence or presence of adenosine and AMP (Table 3). Although the production of 1-MeAde by follicle cells is markedly dependent on GSS concentration (Sano and Kanatani, 1980; Mita *et al.*, 1987), AMP and adenosine at a concentration of 2 mM had no effect on GSS-induced 1-MeAde production (Fig. 4).

Previous studies have shown that if follicle cells are washed with seawater containing low concentrations of Ca^{2+}, the GSS-dependent production of 1-MeAde is markedly decreased (Sano and Kanatani, 1980; Mita, 1994). To determine whether ATPDase activity is influenced by treatment with low Ca^{2+} concentration, the cells were washed with seawater

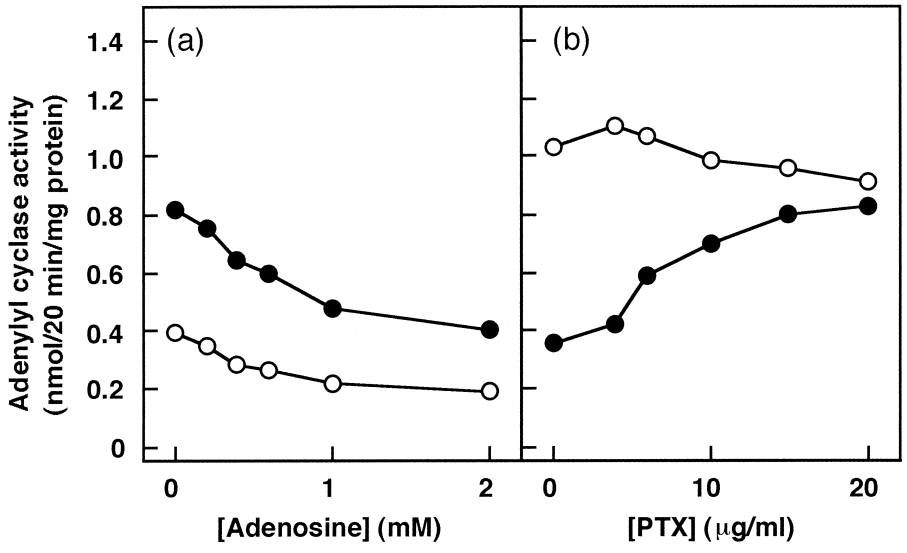

Fig. 3. Effect of adenosine on adenylyl cyclase activity in starfish follicle cells (a) and its reversal by pertussis toxin (b). (a) Crude follicle cell membranes (0.05 mg protein) were incubated for 20 min at 20°C in a reaction mixture (0.1 ml) in the presence of various concentrations of adenosine with (●) or without (○) 0.1 mM GTP. (b) Crude membranes (0.05 mg protein) were incubated for 20 min at 20°C in a reaction mixture (0.1 ml) containing 1 mM NAD^+ and 1 mM DTT in the presence of various concentrations of activated PTX with (●) or without (○) 1 mM adenosine. The values shown are means for duplicate determinations.

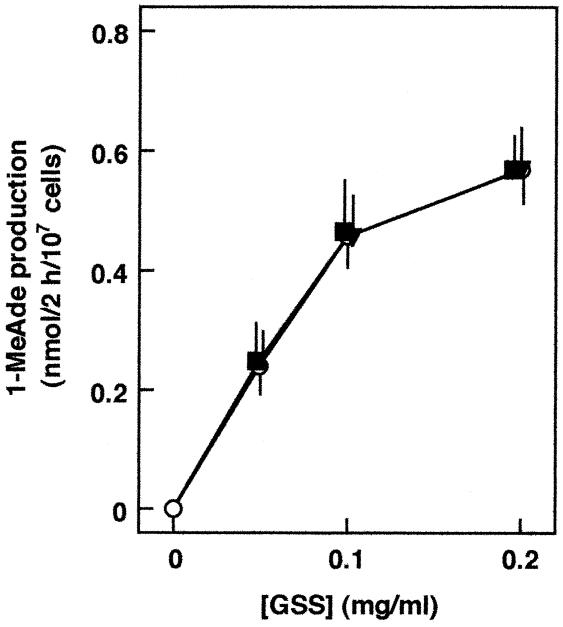

Fig. 4. Effect of AMP and adenosine on GSS-induced 1-MeAde production in starfish follicle cells. The follicle cells (10^7 cells) were incubated in 1.0 ml of ASW containing GSS at the indicated concentrations in the absence (○) and presence of either 2 mM AMP (■) or adenosine (▼). Each value is the mean of three separate experiments. Vertical bars show SEM.

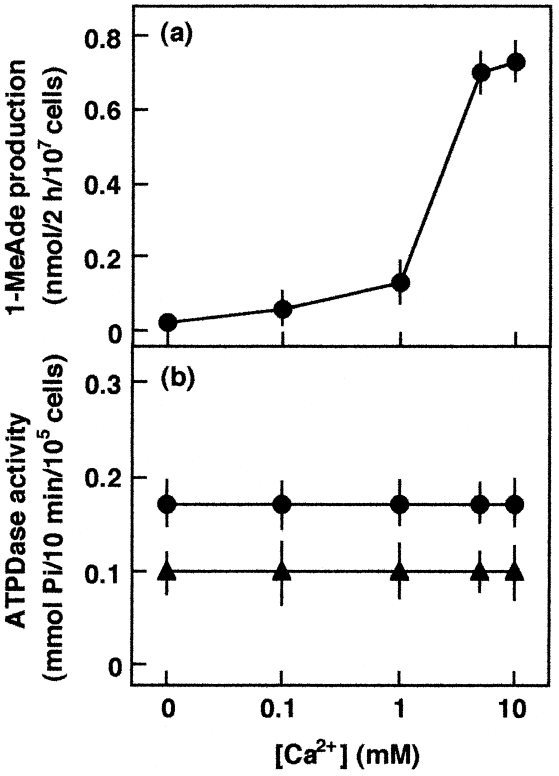

Fig. 5. 1-MeAde production (a) and ecto-ATPDase activity (b) in starfish follicle cells treated with seawater at low concentrations of Ca^{2+}. (a) The follicle cells (10^7 cells/ml) were treated with seawater containing Ca^{2+} at the indicated concentrations and incubated with ASW in the presence of GSS (0.1 mg/ml) for 2 hr at 20°C. After incubation, the cell suspension was centrifuged at 1,000×*g* for 1 min, and the supernatant was used for 1-MeAde assay. (b) The cells (5×10^5 cells/0.1 ml) treated with seawater containing Ca^{2+} at the indicated concentrations were incubated with ASW in the presence of 2 mM ATP (●) or ADP (▲) for 10 min at 20°C. After incubation and centrifugation, the supernatant was used for Pi assay. Each value is the mean of three separate experiments. Vertical bars show SEM.

in the absence or presence of 0.1, 1 5 or 10 mM Ca^{2+}, prepared by substituting NaCl for $CaCl_2$. During incubation with GSS (0.1 mg/ml) for 2 hr at 20°C, maximal 1-MeAde production was obtained in the presence of 10 mM Ca^{2+}, which is the normal concentration in seawater. The amount of 1-MeAde produced decreased in parallel with the decrease in Ca^{2+} concentration (Fig. 5a). In contrast to 1-MeAde production, the activity of ATPDase remained constant in follicle cells washed with seawater containing reduced concentrations of Ca^{2+} (Fig. 5b). It does not appear that ecto-ATPDase activity is correlated with GSS-induced 1-MeAde production.

It has been shown that NaF and AppNHp inhibit starfish ATPDase (Mita *et al.*, 1998). Ecto-ATPDase activity was reduced to about 60% and 50% by 5 mM NaF and 2.5 mM AppNHp, respectively (Table 4). However, these inhibitors had no effect on GSS-induced 1-MeAde production. In addition, neither adenine nucleotides nor Pi had any effect on 1-MeAde-induced oocyte maturation in starfish (data not shown). It is thus unlikely that ecto-ATPDase and its metabolites are directly associated with 1-MeAde production and oocyte maturation.

Table 4. Effect of NaF and AppNHp on 1-MeAde production and ecto-ATPDase activity in starfish follicle cells

Conditions	1-MeAde production* (nmol/2 h/10^7 cells)	ATPDase activity (μmol Pi released/10 min/5×10^6 cells)	
		ATP (2mM)	ADP (2mM)
Control	1.02±0.05	0.20±0.01	0.13±0.01
NaF (5mM)	1.02±0.08 (100)	0.12±0.01 (60)	0.08±0.01 (61)
AppNHp (2.5mM)	1.02±0.07 (100)	0.10±0.01 (50)	0.06±0.01 (46)

*In these experiments, GSS at concentration of 0.1 mg/ml was added to the incubation media. Values are means ±SEM of three separate experiments. Values in parentheses are percentages relative to the control value.

DISCUSSION

The present study showed that a high activity of ecto-ATPDase was present on the surface of starfish ovarian follicle cells. However, we could not obtain direct evidence that the ecto-ATPase is related to GSS-induced 1-MeAde production. It has been demonstrated that the action of GSS in inducing 1-MeAde production depends on interaction with its receptor on the follicle cell surface (Mita and Nagahama, 1991). Since ecto-ATPDase is distributed on the follicle cell surface, it was important to clarify the relationship between ecto-ATPDase and the receptor protein for GSS. It has been reported that an extracellular Ca^{2+} concentration of more than 5 mM is required for maximal production of 1-MeAde through the action of GSS (Mita, 1994). GSS may be unable to bind to its receptor on the follicle cell surface after treatment with seawater containing a low concentration of Ca^{2+} because of decrease or denaturation of its receptors. In this study, the activity of ecto-ATPDase remained constant after treatment of follicle cells with seawater containing low concentrations of Ca^{2+}, whereas GSS-induced 1-MeAde production was markedly decreased. In addition, NaF and AppNHp, which are inhibitors of ATPDase, did not have any effect on GSS-induced 1-MeAde production in follicle cells. GSS could induce 1-MeAde production regardless of the absence or presence of AMP and adenosine, which are metabolites of ATPDase. Thus, ecto-ATPDase and its metabolites do not appear to be involved in 1-MeAde production, suggesting that ecto-ATPDase does not act as a receptor protein for GSS.

Previous studies have shown that two types of G-proteins, Gs and Gi, are present in starfish ovarian follicle cells (Mita and Nagahama, 1991; Mita, 1994). Gs is activated by receptor-mediated GSS action, resulting in the activation of adenylyl cyclase and cyclic AMP formation. In this study, adenosine and AMP were shown to induce a decrease in intracellular cyclic AMP level. It is possible that AMP produced by ecto-ATPDase is further hydrolyzed by a phosphatase to adenosine. This study also showed that adenosine inhibited adenylyl cyclase activity in follicle cells. It is noteworthy that adenosine-induced inhibition of adenylyl cyclase was completely reversed by PTX. This suggests that adenosine inhibits adenylyl cyclase activity via interaction with Gi. It has been demonstrated that during oogenesis, small oocytes in *A. pectinifera* have no competence to 1-MeAde (Bulet *et al.*, 1985). If 1-MeAde is produced before the oocyte maturation stage, these growing oocytes would not undergo maturation and fertilizable. It thus may be possible that adenosine plays a role in inhibition of 1-MeAde production before the maturation stage, since an increase in the level of cyclic AMP is important for the production of 1-MeAde in follicle cells (Mita *et al.*, 1987, 1988, 1989). At the maturation stage, GSS can stimulate 1-MeAde production in follicle cells regardless of the presence of AMP or adenosine.

On the other hand, it was reported that mammalian and avian ATPDases show high homology with CD39, a molecule originally characterized as a lymphoid surface antigen (Handa and Guidotti, 1996; Kaczmarek *et al.*, 1996; Wang and Guidotti, 1996). Since each oocyte in starfish ovaries is surrounded by follicle cells (Schroeder, 1971; Schroeder *et al.*, 1979), ecto-ATPDase on starfish ovarian follicle cells may protect oocytes from phagocytes and parasites during oogenesis.

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