

## [COMMUNICATION]

## Effect of ATP $\gamma$ S on Fertilization Envelope Elevation of Sand Dollar Eggs

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**ABSTRACT**—ATP $\gamma$ S (adenosine 5'-0-(3-thiotriphosphate)) inhibited cortical granule exocytosis of the sand dollar (*Clypeaster japonicus*) egg at insemination when injected before insemination at concentrations of 33–600  $\mu$ M in the cytoplasm. Accordingly the fertilization envelope did not elevate, whereas many sperm were incorporated into the egg and then sperm asters formed. After treatment with Ca ionophore A23187 the fertilization envelope did not elevate from eggs injected with ATP $\gamma$ S, although the threshold concentration of ATP $\gamma$ S to inhibit fertilization envelope elevation was higher than that at insemination. These results suggest that ATP $\gamma$ S would reduce Ca<sup>2+</sup>-sensitivity of cortical granules by means of thiophosphorylation and, therefore, would prevent a transient increase in intracellular free Ca<sup>2+</sup> concentration at insemination or parthenogenetic activation from inducing cortical granule exocytosis. Another analog, AMPPNP (5'-adenylylimido diphosphate) (< 600  $\mu$ M in the egg cytoplasm) did not inhibit fertilization envelope elevation at insemination. However, ATP $\gamma$ S and AMPPNP at 900  $\mu$ M or more in the egg cytoplasm induced the envelope elevation by the injection. AMPPCP (5'-adenylylmethylene diphosphate) showed no effect.

### INTRODUCTION

ATP is the fundamental molecule as an energy source for cellular activities, especially for such as the generation of force and movement, the synthesis of biological molecules, and the active transport of molecules. ATP is used as a substrate for more than 100 enzymes not only to utilize high-energy bonds of phosphates by hydrolysis, but also to modify enzymes by phosphorylation in order to

control their activity. Many analogs of ATP have been synthesized and used in order to investigate properties of enzymes [1]. ATP $\gamma$ S (adenosine 5'-0-(3-thiotriphosphate)), one of phosphate analogs of ATP may be used in place of ATP by kinases, whereas enzymes modified covalently in thiophosphorylated forms by kinases are not favored substrates for phosphatases and, therefore, ATP $\gamma$ S can possibly control the activities of key enzymes and change cellular activity irreversibly [2]. On the other hand, AMPPCP (5'-adenylylmethylene diphosphate) or AMPPNP (5'-adenylylimido diphosphate), which has non-hydrolyzable linkage between  $\beta$  and  $\gamma$  phosphorus, is not a suitable substrate for any of kinases or any of enzymes which cleave the  $\beta$ - $\gamma$  linkage of ATP [1] and may affect cellular activities in a manner different from ATP $\gamma$ S.

In the present study, I investigated the effect of these ATP analogs on fertilization of sand dollar eggs in order to understand the role of phosphorylation in fertilization by means of microinjection because they might not be uptaken easily by the eggs from the surrounding medium. Only ATP $\gamma$ S inhibited cortical granule exocytosis at insemination, but it did not inhibit sperm incorporation or aster formation, suggesting that ATP $\gamma$ S might make cortical granules insensitive to Ca<sup>2+</sup> through thiophosphorylation.

### MATERIALS AND METHODS

I obtained gametes of the sand dollar, *Clypeaster japonicus*, by the injection of 0.5 M KCl or sea



water containing 1 mM acetylcholine into the coelomic cavity. The Ca ionophore, A23187 was dissolved at 10 mM in dimethylsulfoxide, diluted to 10 or 20  $\mu$ M in artificial sea water (Jamarin, Jamarin Lab. Osaka), and used for parthenogenetic activation. Two to 50 mM of ATP $\gamma$ S, 25–100 mM AMPPNP, and 25 mM AMPPCP dissolved in 50 mM MOPS (3-(N-morpholino) propanesulfonic acid) (pH. 7.0) were used for injection. 100–400 mM LiCl and 25–100 mM ATP dissolved in 50 mM MOPS (pH. 7.0) were used as controls.

I carried out microinjection at  $25 \pm 1^\circ\text{C}$  as

described by Hiramoto [3]. The final concentrations of ATP analogs in the egg cytoplasm were calculated as follows. The injected amount of ATP analogs (the injected volume of the solution multiplied by the concentration of them) was divided by the egg volume of 0.8 nl [4].

## RESULTS

The effect of ATP $\gamma$ S on fertilization or activation of the sand dollar (*Clypeaster japonicus*) egg is summarized in Figure. 1 when ATP $\gamma$ S was injected

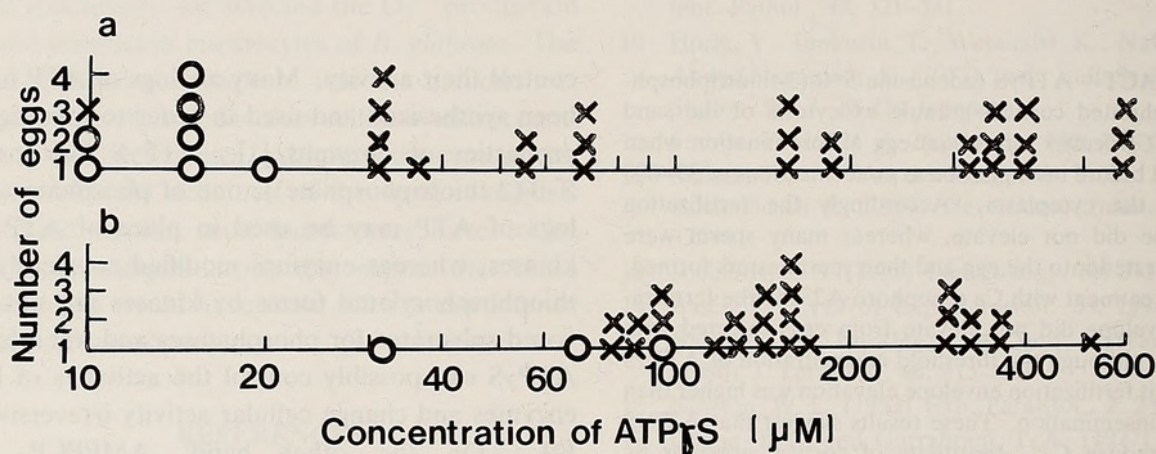


FIG. 1. Effect of ATP $\gamma$ S injection into unfertilized eggs on fertilization envelope elevation at insemination (a) and activation with A23187 (b). Abscissa; final concentrations of ATP $\gamma$ S injected into the eggs. o; eggs with the fertilization envelope at insemination or by treatment with A23187. x; eggs without the envelope after insemination or by the treatment. The eggs were counted in case of a when one or more sperm asters were observed in these eggs after insemination.

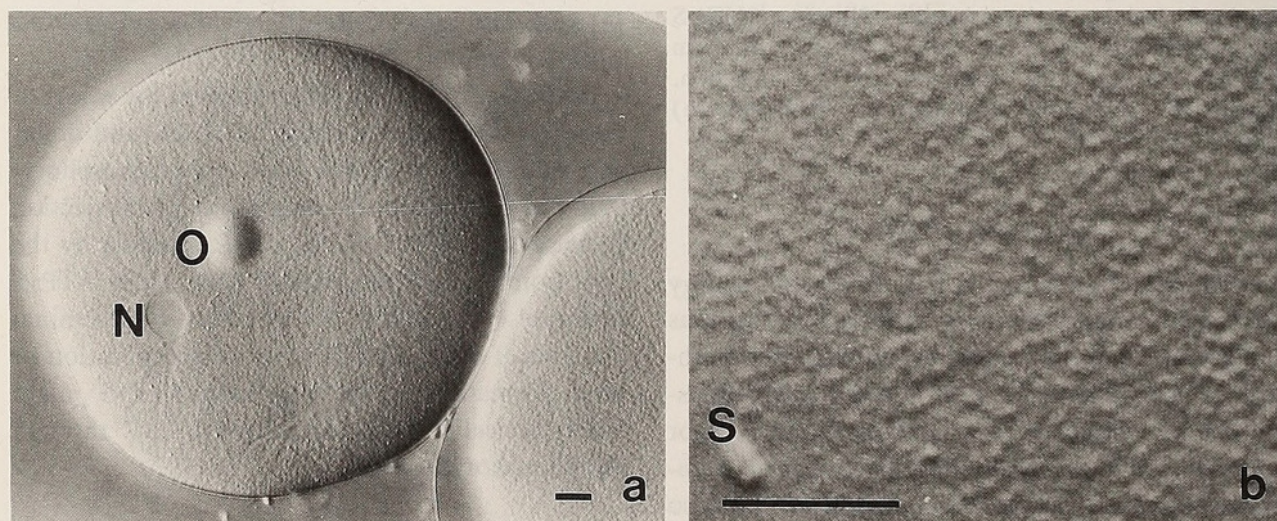


FIG. 2. An egg which was injected with ATP $\gamma$ S and inseminated after injection. a; three asters are found in this field. O indicates an oil drop introduced at the time of injection, and N synkaryon. b; the cortex of the same egg in a. S indicates a sperm head on the egg surface. It is noted that none of cortical granules disappeared. Bar shows 10  $\mu$ m.



at 600  $\mu$ M or less in final concentration into the unfertilized one. No significant effect of ATP $\gamma$ S on egg morphology was observed. However, when the eggs were inseminated after injection of ATP $\gamma$ S at 33–600  $\mu$ M in the cytoplasm, the fertilization envelope did not elevated from any of 26 injected eggs (Fig. 1a). In these eggs, sperm were incorporated and sperm asters were observed (Fig. 2a) although cortical granules appeared intact (Fig. 2b), and the cleavage furrow was developed in some eggs. Seven out of 8 eggs injected with ATP $\gamma$ S at 20  $\mu$ M or less showed fertilization envelope elevation at insemination and developed normally. Occurrence of sperm aster formation suggests that intracellular  $\text{Ca}^{2+}$  increased in the cytoplasm of the eggs injected with ATP $\gamma$ S at sperm incorporation.

In order to confirm this possibility, the eggs injected with ATP $\gamma$ S were treated with Ca ionophore which may increase the intracellular  $\text{Ca}^{2+}$  and result in fertilization envelope elevation [8]. When 30 eggs injected with ATP $\gamma$ S at 600  $\mu$ M or less in final concentration were incubated in sea water containing 10 or 20  $\mu$ M A23187, the fertilization (activation) envelope did not elevate from 27 out of 28 eggs injected with ATP $\gamma$ S at 80  $\mu$ M or more (Figs. 1b and 3), but the fertilization envelope elevated from both of 2 eggs injected with ATP $\gamma$ S at 70  $\mu$ M or less. These results mean that ATP $\gamma$ S inhibited fertilization envelope elevation, although intracellular  $\text{Ca}^{2+}$  concentration increased after treatment with Ca ionophore and

that the threshold concentration of ATP $\gamma$ S to inhibit the elevation after the treatment was higher than that at insemination.

The fertilization envelope elevated from all of 6 eggs injected with ATP $\gamma$ S at 900  $\mu$ M or more in final concentration in the egg cytoplasm shortly after injection without insemination.

Fertilization including the fertilization envelope elevation normally occurred by insemination in 6 eggs injected with AMPPNP at 600  $\mu$ M or less in final concentration in the egg cytoplasm. On the other hand, the fertilization envelope elevated from all of 8 eggs injected with AMPPNP at 900  $\mu$ M or more in final concentration shortly after injection. No effect was observed when AMPPCP was injected into 5 eggs up to 2000  $\mu$ M in the egg cytoplasm and all of them were fertilized normally after insemination.

ATP analogs may deplete some inorganic ions, especially such as  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  in the egg cytoplasm because ATP and these analogs chelate them [14] and  $\text{Li}^+$  was introduced into the eggs at ATP $\gamma$ S, AMPPNP, and AMPPCP injection because these analogs were purchased as such  $\text{Li}^+$  salt as  $\text{Li}^+$  ATP $\gamma$ S, which might possibly cause some side effects on fertilization of sand dollar eggs. ATP and LiCl were injected into 4 unfertilized eggs at the concentration of up to 1.6 mM in the egg cytoplasm and 10 eggs at the concentration of up to 19 mM as control, respectively. No significant effect was observed after injection and all of the injected eggs were fertilized normally after insemination.

## DISCUSSION

ATP $\gamma$ S inhibited the exocytosis of cortical granules of sand dollar eggs at insemination when it was injected before insemination at low concentration in the cytoplasm, and, accordingly the fertilization envelope did not elevate, although sperm could enter the eggs and sperm asters formed. Occurrence of sperm aster formation indicates  $\text{Ca}^{2+}$  increase in the egg cytoplasm by two reasons as follows. First, it is well-known that  $\text{Ca}^{2+}$  concentration increases transiently in the cytoplasm of fertilized eggs where sperm aster forms [5, 6]. Secondly, EGTA which can maintain in-

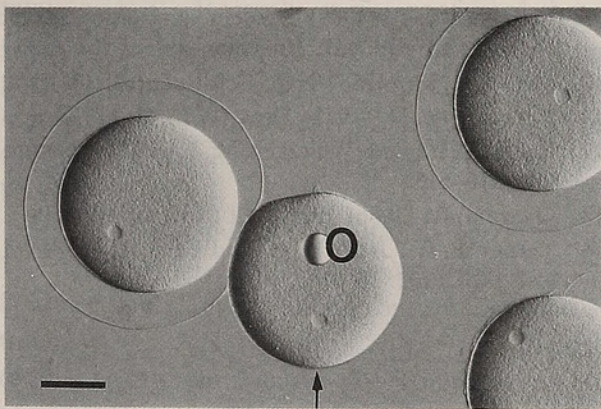


FIG. 3. An egg indicated by arrow which was injected with ATP $\gamma$ S and treated with 10  $\mu$ M A23187 did not induce fertilization envelope elevation, but the other eggs did. Bar shows 50  $\mu$ m.



tracellular free  $\text{Ca}^{2+}$  concentration at such a low level as that in unfertilized eggs by means of injection did not inhibit sperm entrance, but inhibited both cortical granule exocytosis and sperm aster formation [4, 7].  $\text{ATP}\gamma\text{S}$  also inhibited cortical granule exocytosis of the injected eggs after treatment with the Ca ionophore, which is known to increase intracellular  $\text{Ca}^{2+}$  concentration [8]. It is inferred from these facts that  $\text{ATP}\gamma\text{S}$  prevents  $\text{Ca}^{2+}$  increase from inducing cortical granule exocytosis, though the increase in the cytoplasm of the eggs injected with  $\text{ATP}\gamma\text{S}$  occurs both after insemination and by treatment with the Ca ionophore.

Effective concentration of  $\text{ATP}\gamma\text{S}$  was much lower in the egg cytoplasm than the cytoplasmic concentration of ATP, which is a few mM [9]. AMPPNP and AMPPCP, which are possible competitive inhibitors of ATP, showed no effect on fertilization envelope elevation at low concentration during fertilization or activation. These facts suggest that  $\text{ATP}\gamma\text{S}$  was used not as a substrate for hydrolases but as a substrate for kinases in the egg cytoplasm and that the thiophosphorylated products might reduce  $\text{Ca}^{2+}$ -sensitivity of cortical granules and inhibit cortical granule exocytosis.

The inhibitory effect of  $\text{ATP}\gamma\text{S}$  on fertilization envelope elevation resembles that of local anesthetics such as procaine and urethane [10, 11]. Procaine raised the effective concentration of  $\text{Ca}^{2+}$  in the egg cytoplasm to elevate the fertilization envelope at the injection of Ca buffers up to such an extremely high level as  $40\ \mu\text{M}$  [4]. Reduction of ATP by treatment with metabolic inhibitors also inhibited cortical granule exocytosis after insemination [12, 13], suggesting that the reduction might lower  $\text{Ca}^{2+}$ -sensitivity of cortical granules [12]. It has not yet been known whether or not these reagents inhibited the exocytosis through phosphorylation.

At millimolar concentration,  $\text{ATP}\gamma\text{S}$  and AMPPNP induced cortical granule exocytosis

when injected into sand dollar eggs. It is unknown what kinds of reaction in the egg cytoplasm they would let induce the exocytosis because they may affect enzymes in the cell as reviewed by Yount [1]. However, one of possibilities is that these two analogs would mimic  $\text{GTP}\gamma\text{S}$  (guanosine 5'-O-(3-thiotriphosphate)) by activating a GTP-binding protein because  $\text{GTP}\gamma\text{S}$  cause fertilization envelope elevation of sea urchin eggs after injection [14].

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