and pPGCs in some anuran species[5-7]. In contrast, SBA, Con A and GS-I had a high affinity for granules in specific regions. These granules were distributed widely on the ventral side of the mid-gut and appeared on or among yolk cells, while some appeared to collect in a few cells around the mid-gut. Their shape and distribution closely resembled those of PAS-positive granules in the embryos of this species and of *R. brevipoda* at the same stage[3]. Therefore, these lectin-affinity granules may migrate separately from cells at the far ventral side of the mid-gut toward cells on the dorsal side, in view the behavior of PAS-positive granules during embryogenesis. This possibility is discussed below.

The second question concerns a possible correlation between the effects of lectins and the number of PGCs. The lectins with no affinity for granules had no effect on the formation of PGCs. In contrast, the other lectins had a more or less inhibitory effect. In particular, Con A and GS-I were very effective, in parallel to their high affinity for the specific granules. A similar effect of lectins was demonstrated in another experiment (injection of embryos with a fraction of granules prepared by centrifugation): granules pre-treated with GS-I did not contribute at all to the proliferation of PGCs, unlike the granules pre-treated with PNA or MPA (unpublished data).

The reduction in the size of PGCs in experimental animals was also of interest, since such a phenomenon has been reported in anuran larvae derived from UV-irradiated or overripe eggs[8-10]. In the latter, it was suggested that the alterations in PGCs were the result of the inhibitory migration of germ plasm during cleavage stages. Therefore, the present results suggest that the alterations in PGCs, in terms both of number and of size, may have resulted from an impediment in the normal migration of functional granules due to the lectins. If such is the case, it is possible that the lectin-affinity granules include some determinant of germ cells and are directly involved in the formation of PGCs. In other words, it is likely in this species, that the determinants leave the cells in which they remained during the blastula stage and move separately toward a new position, through yolk cells, through the use of an appropriate antigen on their envelopes. Given the results obtained from the PNA-treated group, it is probable, in *R. nigromaculata*, that the PGCs are not determined at earlier embryonic stages, such as the blastula stage. This suggestion is in contrast to the general assumption about the determination of PGCs in anura. It may be difficult to purify the target granules from other granules because many lectins have some affinity for yolk platelets. In particular, Con A, SBA and WGA had considerable affinity for yolk platelets, although it is uncertain whether they have the same affinity for small yolk platelets that have been digested to a large extent. If the lectins have only weak affinity for small yolk platelets, lectin-coated beads should facilitate isolation of the target granules, since large yolk platelets are not included in the granular fraction obtained by centrifugation (crude mitochondria).

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0014-4754/91/010097-04$1.50 + 0.20/0

Involvement of a sperm aminopeptidase in fertilization of the sea urchin

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Received 10 November 1989; accepted 26 July 1990

Summary. Inhibitory efficiencies of bestatin methyl ester and its nine analogs for sea urchin sperm aminopeptidase activity were similar to the efficiency of the same compounds as inhibitors either of sperm respiration or of fertilization. This suggests that a sperm aminopeptidase plays a role in fertilization in the sea urchin, possibly through its involvement in sperm respiration.
Key words. Aminopeptidase; bestatin; fertilization; respiration; sperm; sea urchin.

Some proteases of spermatozoa and eggs are thought to be involved in fertilization in echinoderms. Several lines of evidence have indicated that a chymotrypsin-like enzyme of the sperm is a lytic agent[3,4] (lysin), which allows the sperm to penetrate through the vitelline coat of the eggs, and that a trypsin-like enzyme of the eggs partici-

pates in elevation of the fertilization envelope and establishment of the block to polyspermy[5-8]. However, the role of aminopeptidase in fertilization has not been reported so far in echinoderms, although some properties of the enzyme have been examined in bull sperm[9].

We previously showed that an aminopeptidase exists in sperm of the sea urchin, *Strongylocentrotus intermedius*, and that the enzyme activity in the intact sperm is scarcely inhibited by bestatin, [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine (fig. 1), which is a specific inhibitor of aminopeptidase[11]. The enzyme is, however, strongly inhibited by the ethyl ester derivative of bestatin[12].

The present paper describes the comparison of inhibitory effects of methyl ester derivatives of bestatin, and of nine bestatin analogs, on fertilization in *S. intermedius* and on the respiration and aminopeptidase activity of its sperm.

Materials and methods

Collection of gametes: Eggs and sperm of the sea urchin, *S. intermedius*, were collected by introducing 0.5 M KCl into the coelom. Esterification of bestatin and its analogs: Bestatin and its analogs were generous gifts of Dr. W. Tanaka of Nippon Kayaku Co. Methyl ester derivatives of bestatin and its analogs were prepared by treating them with 5% hydrogen chloride in methanol at room temperature overnight. After evaporation, each residue was dissolved in filtered sea water buffered with 10 mM Tris-HCl, pH 8.0 (FSW) to give a concentration of 1 mM. The complete esterification was confirmed by high-performance liquid chromatography on a reversed phase column.

Inhibition of aminopeptidase activity in sperm: 'Dry' sperm was washed twice by centrifugation (1000 × g, 5 min) with 10 volumes of artificial sea water (ASW: 460 mM NaCl, 10 mM $CaCl_2$, 10 mM KCl, 50 mM $MgCl_2$). The washed spermatozoa were suspended in 10 volumes of ASW. The sperm suspension in a volume of 20 µl was preincubated in 0.5 ml of ASW that was buffered with 10 mM Tris-HCl (pH 8.0) for 15 min at 0 °C in the presence or absence of a certain concentration of an inhibitor. The aminopeptidase reaction was started by adding 20 µl of 0.5 mM leucine-4-methylcoumaryl-7-amide (Leu-MCA, the Peptide Institute, Osaka, Japan) and the mixture was allowed to stand for 30 min at 25 °C. The enzyme activity was determined by measuring the fluorescence due to one of the reaction products, 7-amino-4-methylcoumarin, with excitation at 380 nm and emission at 460 nm using a Shimazu spectrofluorophotometer (model RF-500).

Inhibition of fertilization: A suspension of about 100 eggs in a volume of 25 µl was mixed with 0.4 ml of FSW containing a certain concentration of an inhibitor in a multidish. The eggs were inseminated at 15 °C with 25 µl of sperm suspension (1.6×10^6 cells/ml), the concentration of which was slightly higher than the minimum for 100% fertilization in the absence of the inhibitor. Fertilization ratio was determined at 30 min after insemination by measuring the number of eggs in which the elevation of the fertilization envelope had been confirmed.

Inhibition of sperm respiration: The sperm suspension in a volume of 10 µl was added to 2 ml of FSW containing a certain concentration of an inhibitor at 15 °C. Respiration rates of sperm were determined after the rates had become constant (about 3 min after the addition) by using a YSI Biological Oxygen Monitor (Model 53, Yellow Spring Instrument Co.) equipped with a temperature control chamber. In all determinations of enzyme activity, fertilization ratio or respiration, the inhibitors were used after they had been diluted with ASW or FSW to the appropriate concentration. Each inhibitor seemed to be soluble at the concentrations used.

Results and discussion

Our previous work[12] demonstrated that aminopeptidase activity in intact *S. intermedius* sperm was strongly inhibited by bestatin ethyl ester, but was scarcely affected by bestatin itself (fig. 1). The two compounds, however, were almost indistinguishable from each other in their potency as aminopeptidase inhibitors, when the enzyme solubilized from the sperm was tested. In this study, methyl ester derivatives of bestatin and of nine analogs were compared with respect to their inhibitory effects on the activity of sperm-associated aminopeptidase, fertilization, and the respiration of sperm of *S. intermedius*. The results are presented in the table. Among the ten compounds examined, the derivatives of bestatin and three of its analogs (I, II, III, shown in the table) were found to show strong inhibitory effects on all the three subjects. The derivatives of other analogs (IV–IX) exhibited little effects on any of them. Reproducible results were obtained with different batches of spermatozoa.

Figures 2 and 3 show details of the comparison among the four effective compounds. As an inhibitor for the aminopeptidase activity, analog I methyl ester has almost the same potency as that of bestatin methyl ester, and is superior to methyl esters of analogs II and III (fig. 2). The former two compounds were also more effective than the latter two in the inhibition of respiration (fig. 3A) and of fertilization (fig. 3B). Analog II ester was the second strong inhibitor, and analog III ester was

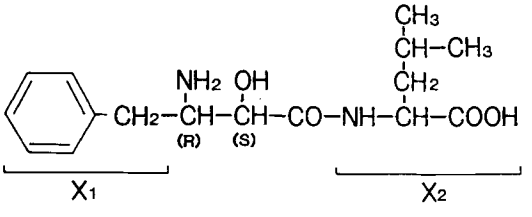

Figure 1. Structure of bestatin[10]. Analogs shown in the table are those substituted at X_1 and X_2 residues as indicated. The derivatives used were esterified at their carboxyl termini.

Effects of methyl ester derivatives of bestatin and its analogs on sperm aminopeptidase activity, sperm respiration, and fertilization of the sea urchin, *S. intermedius*

Analog	3-Amino-2-hydroxy-X_1-butanoyl-X_2-OMe X_1	X_2	Enzyme activity[a]	Respiration[b]	Fertilization[c]
None			100%	100%	100%
B[d]	-4-phenyl-	Leu	5	2	0
(I)	-4-p-nitrophenyl-	Leu	5	4	0
(II)	-4-cyclohexyl-	Leu	10	5	0
(III)	-4-p-chlorophenyl	Leu	10	15	5
(IV)	-4-phenyl-	Gly	94	71	100
(V)	-4-phenyl-	βAla	78	91	100
(VI)	-4-phenyl-	Pro	91	107	100
(VII)	-4-phenyl-	Gln	82	98	100
(VIII)	-4-phenyl-	Ser	91	104	100
(IX)	-	Leu	86	94	100

[a] The concentration of analogs tested was 10 μM. [b] The concentration of analogs tested was 0.5 mM. [c] The concentration of analogs tested was 1 mM. [d] Bestatin methyl ester.

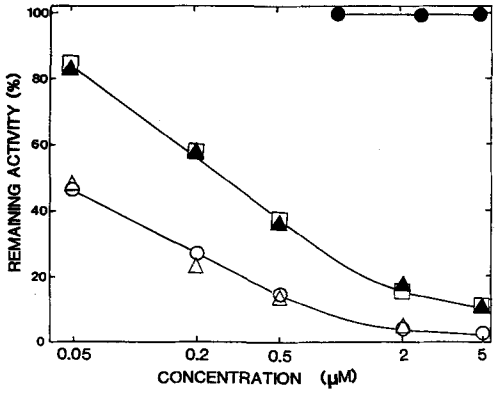

Figure 2. Effects of methyl ester derivatives of bestatin and of its three analogs on sperm aminopeptidase activity of the sea urchin, *S. intermedius*. The sperm suspension was preincubated for 15 min in 0.5 ml of ASW buffered with 10 mM Tris-HCl (pH 8.0) containing the inhibitor indicated, and the reaction was started by adding 20 μl of 0.5 mM Leu-MCA. After the reaction for 30 min at 25 °C, the remaining activity was determined fluorometrically. ●, bestatin; ○, bestatin methyl ester; △, methyl ester of analog I; □, methyl ester of analog II; ▲, methyl ester of analog III.

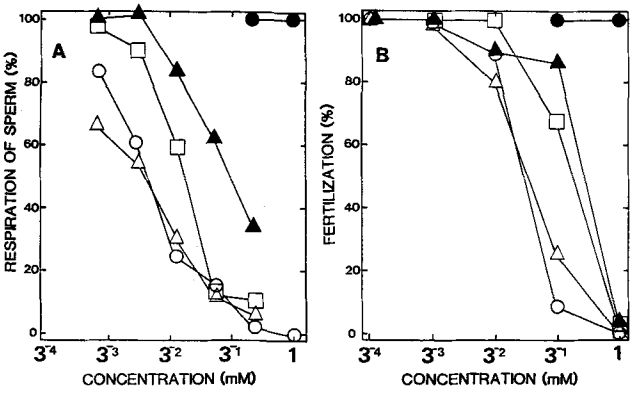

Figure 3. Effects of methyl ester derivatives of bestatin and of its three analogs on sperm respiration (A) and fertilization (B) of the sea urchin, *S. intermedius*. *A* The sperm suspension was incubated at 15 °C in 2 ml of filtered sea water buffered with 10 mM Tris-HCl (pH 8.0) containing the inhibitor indicated, and respiration rates of sperm were determined. *B* Eggs (100 cells) were inseminated in 0.4 ml of filtered sea water buffered with 10 mM Tris-HCl (pH 8.0) containing the inhibitor indicated. Fertilization ratio was determined at 30 min after insemination, by measuring the number of eggs in which elevation of the fertilization envelope had been confirmed. ●, bestatin; ○, bestatin methyl ester; △, methyl ester of analog I; □, methyl ester of analog II; ▲, methyl ester of analog III.

the weakest in both cases. Reproducible results were again obtained with different batches of spermatozoa. Thus, the rankings among the four inhibitors were similar to their ranking for potency as inhibitors of aminopeptidase activity, suggesting the participation of aminopeptidase in respiration and fertilization.

However, the concentration of the inhibitors required for 50% inhibition of either of the two 'biological activities', respiration and fertilization, was approximately three orders of magnitude higher than that required for 50% inhibition of the enzyme activity. This difference seems to be explicable by the difference between the ratio (K_m value/concentration) of the natural substrate, presumed to be responsible for the biological activities, and that of the synthetic substrate used for determination of aminopeptidase activity. The concentration of the synthetic substrate, Leu-MCA, was fixed at 19 μM in the assay medium, and its K_m value (for the enzyme in a purified form) is known to be 5.4 μM at pH 7.5 and 25 °C [13]. When the natural substrate is fully characterized, therefore, it will be possible to verify the above explanation.

The possibility that methyl ester derivatives of bestatin and its analogs owed their inhibitory effects on fertilization to their cationic detergent-like properties was denied by the following experiments. When sperm suspension pretreated with the 0.5 mM methyl ester of bestatin, analog I, or analog II, was diluted 17-fold with filtered sea water in order to decrease the inhibitor concentration to an ineffective level (0.03 mM) and then used for insemination, the eggs were fertilized as efficiently as those inseminated in the absence of inhibitor. The result indi-

cates that the inhibitory effect of the compounds is reversible. On the other hand, the inhibitory effect of a typical cationic detergent, dodecylbenzyltrimethylammonium chloride, was irreversible. Sperm inactivated by pretreatment with the detergent at 0.5 mM was unable to recover its ability to fertilize the eggs even when the detergent concentration was diluted 17-fold. Thus the mechanism of the inhibitory effect of methyl ester derivatives of bestatin and its analogs seems to be different from that of the cationic detergent.

Christen et al.[14,15] have reported that both respiration and motility of sea urchin sperm were inhibited when the intracellular pH was decreased. In a preliminary experiment, we observed that motility of the sperm was inhibited by the addition of bestatin methyl ester. The accumulation in the sperm cell of methyl ester derivatives of bestatin and its analogs, which are cationic in nature like NH_4^+ under physiological conditions, would not decrease the intracellular pH, because NH_4^+ has been reported to elevate this pH[14,15]. It seems probable, therefore, that the action of the compounds used in this study on sperm respiration and motility, resulting in the depression of fertilization, is due to their inhibitory effect on sperm aminopeptidase. Further study will be necessary in order to elucidate the precise function of aminopeptidase in the sperm.

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2 Acknowledgment. We are grateful to Dr Wataru Tanaka of Nippon Kayaku Co. for his gifts of bestatin and its analogs. We are also indebted to Mrs Akiko Tsuchida-Watanabe for her technical assistance, and to the staff of Asamushi Marine Biological Station, Tohoku University, where part of this work was carried out. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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0014-4754/91/010100-04$1.50 + 0.20/0

Gonyauline: A novel endogenous substance shortening the period of the circadian clock of a unicellular alga

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Received 6 March 1990; accepted 4 May 1990

Summary. The circadian clock in the unicellular alga *Gonyaulax polyedra* is accelerated by a substance in extracts from the cells themselves. The extracts have been fractionated using the circadian rhythm of bioluminescence as bioassay. The active substance, termed gonyauline, has been isolated and characterized as a novel low molecular weight cyclopropanecarboxylic acid (*S*-methyl-*cis*-2-(methylthio) cyclopropanecarboxylic acid). Synthetic gonyauline has a similar shortening effect on the period of the circadian clock.
Key words. Circadian rhythm; period length; creatine; *S*-methyl-*cis*-2-(methylthio) cyclopropanecarboxylic acid; *Gonyaulax polyedra.*

Many biological phenomena occur predominantly at a certain time of day. In the unicellular marine dinoflagellate, *Gonyaulax polyedra*, several functions fall into this category. For example, cell division, photosynthesis, bioluminescence, motility and pattern formation show circadian rhythmicity which may persist under constant conditions with a precise period of about 24 h (see controls in fig. 1)[1-4]. The length of the free-running circadian period (τ) can be altered by light intensity[5] and by chemicals including propanol extracts of several eukaryotic organisms[6], and these τ-effects can depend on the spectral composition of the background light[7]. We have recently isolated and identified creatine from mammalian muscle as an effective period-shortening substance in *Gonyaulax*[7].

Here we report the isolation and structural identification of an endogenous period-shortening substance obtained from extracts of *G. polyedra*, termed gonyauline. Synthetic gonyauline has a comparable τ-shortening activity. Along with the proteoglycan gene product of the per-lo-