Involvement of prolyl endopeptidase in ascidian fertilization

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Summary. Inhibitory effects on fertilization of the ascidian of three benzyloxycarbonyl(Z)-aminoacyl prolinals and Z-Gly-Pro-chloromethyl ketone added before and after insemination were examined. The results suggest that the prolyl endopeptidase is involved in the process of fertilization, especially in a process taking place between chorion elevation and cell cleavage.
Key words. Fertilization; prolyl endopeptidase; prolinal; ascidian.

Several proteases, such as sperm lysins[2] and egg cortical granule proteases[3], have been reported to be present in spermatozoa and eggs. However, proteases functioning in the process between cortical reaction and the first cell cleavage have not yet been reported, except for the enzymes probably participating in male pronucleus formation[4].

In a previous study[5,6], we reported the presence of prolyl endopeptidase (post-proline cleaving enzyme, EC 3.4.21.26) in the sperm and eggs of the ascidian *Halocynthia roretzi*, which occupies a phylogenetic position between vertebrates and true invertebrates. This enzyme cleaves uniquely at a limited site; the carboxyl side of proline residues in proline-containing peptides. We have designed and synthesized specific inhibitors of this enzyme, containing prolinal at the carboxyl termini[7,8]. In the present report, we examine the inhibitory effect of these inhibitors of prolyl endopeptidase on the fertilization of *H. roretzi* and present evidence for the involvement of this enzyme in the process of fertilization, especially in a process between chorion elevation and cell cleavage.

Materials and methods. Gametes of *H. roretzi* from Mutsu Bay were collected from a pair of gonads with gonoducts, as described previously[9]. Eggs (100–200) were incubated for 1 to 2 min in 1 ml of seawater, filtered and buffered with 10 mM Tris-HCl (pH 8.0), containing various concentrations of inhibitors of proteases and cytoskeletal proteins, and then inseminated at the temperature of seawater at which the ascidian spawns, i.e., 13 °C. Alternatively, the inhibitor was added to the mixture of sperm and eggs at the indicated time after insemination. Percentages of the eggs showing chorion elevation and showing the first cleavage were determined at 30 min and 2.5 h, respectively, after insemination. Colchicine and cytochalasin B were purchased from Nakarai Chemicals (Tokyo) and Sigma Chemical Co. (St. Louis), respectively.

Benzyloxycarbonyl(Z)-Gly-Pro-chloromethyl ketone was a generous gift of Dr T. Yoshimoto and Prof. D. Tsuru of Nagasaki University. Z-aminoacyl prolinals were prepared as described previously[7,8].

Results and discussion. We have previously reported that Z-Gly-Pro-chloromethyl ketone, a specific inhibitor of prolyl endopeptidase[10], inhibits the fertilization of the ascidian *H. roretzi*[5]. Fertilization measured on the basis of cell cleavage was inhibited by this inhibitor at a concentration lower than that required for the inhibition of fertilization measured on the basis of chorion elevation; about 0.1 mM of the inhibitor was required for 50% inhibition using the former criterion, whereas only 20% inhibition was observed at the concentration of 0.5 mM using the latter criterion.

In order to investigate whether the same was true using another type of inhibitor for prolyl endopeptidase, we examined the inhibitory effects on fertilization of Z-Pro-prolinal, Z-Val-prolinal, and Z-Ala-prolinal, which had been previously shown to be transition-state-like inhibitors specific for this enzyme[7,8]. The results are

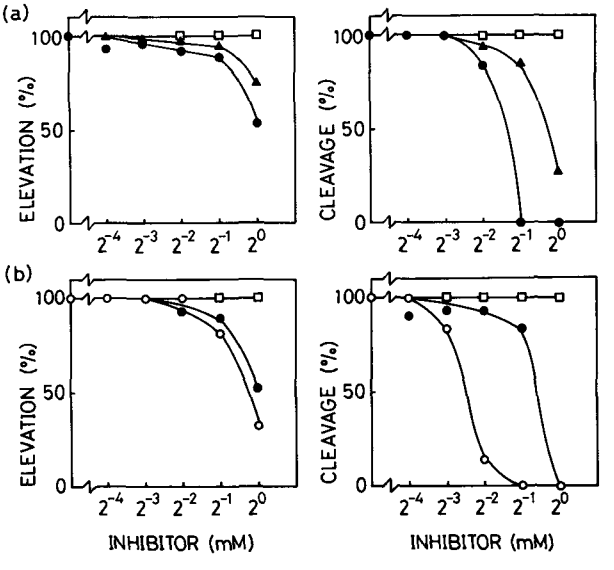

Figure 1. Effects of Z-aminoacyl prolinals on the fertilization of *H. roretzi* measured on the bases of chorion elevation and of cell cleavage. Eggs (100–200) were incubated for 1–2 min in 1 ml of seawater filtered and buffered with 10 mM Tris-HCl (pH 8.0) containing the indicated inhibitor dissolved in 0.5% methanol, and then inseminated. The results obtained with two different batches of gametes are illustrated in *a* and *b*. ○, Z-Pro-prolinal; ●, Z-Val-prolinal; △, Z-Ala-prolinal; □, control (0.5% methanol).

shown in figure 1. It was again found, using each of the three prolinal compounds, that the concentration required for effective inhibition of fertilization measured on the basis of cell cleavage was lower than that required for the inhibition of fertilization measured on the basis of chorion elevation. Z-Pro-prolinal was the most potent inhibitor using either of the criteria for fertilization. The next was Z-Val-prolinal, followed by Z-Ala-prolinal. The ranking of the three compounds by their inhibitory effects on ascidian fertilization was the same as their ranking by their inhibitory effects on the activity of prolyl endopeptidases purified from eggs and from sperm of the same animal[6]. Thus, these results indicate that prolyl endopeptidase functions mainly in the process leading to cell cleavage, and only partly in the process leading to chorion elevation.

In order to prove this proposition for the participation of prolyl endopeptidase in the former process of fertilization, and to define the timing of its action in the process, we examined the time-dependence of the inhibitory effects of Z-Pro-prolinal and Z-Gly-Pro-chloromethyl ketone on the cell cleavage, by adding each of the compounds at the specified times after insemination. As shown in the table, the cleavage was completely suppressed when a fixed amount of each compound was added at the same time as insemination and also at 30–75 min after insemination. The extent of the inhibitory effect was observed to diminish markedly if each compound was added at 90–100 min after insemination. When the concentration of Z-Pro-prolinal added at the same time as insemination or at 30 min after insemination was diluted about 16-fold at 60 min after insemination, the percentage of cell cleavage increased from zero to about 50%. The results suggest that the prolyl endopeptidase functions between 60 and 90–100 min after insemination. Among the inhibitors for cytoskeletal proteins, colchicine (an inhibitor for polymerization of tubulin) at the concentration of 0.2 mg/ml inhibited the cell cleavage in a time-dependent manner similar to that observed in these protease inhibitors, while cytochalasin B (an inhibitor for cytokinesis, i.e. polymerization of actin) at the concentration of 0.01 mg/ml had an effect with no time dependency, throughout the experimental duration.

Effects of prolyl endopeptidase inhibitors added after insemination on fertilization of the ascidian, *H. roretzi*

	Cleavage (%) Time of addition after insemination (min)						
	0	30	60	75	90	100	120
Experiment A							
(1) 1 mM Z-Pro-prolinal	0	0	0		98		94
(2) 0.5% methanol	90	94	97		94		93
Experiment B							
(3) 0.25 mM Z-Gly-Pro-chloromethyl ketone	0	0	0	0		76	95
(4) 2.5% DMSO	93	100	100	95		99	100

Z-Pro-prolinal (1 mM) was dissolved in 0.5% methanol and Z-Gly-Pro-chloromethyl ketone (0.25 mM) was in 2.5% dimethylsulfoxide (DMSO). The first cell cleavage was measured at 2.5 h after insemination.

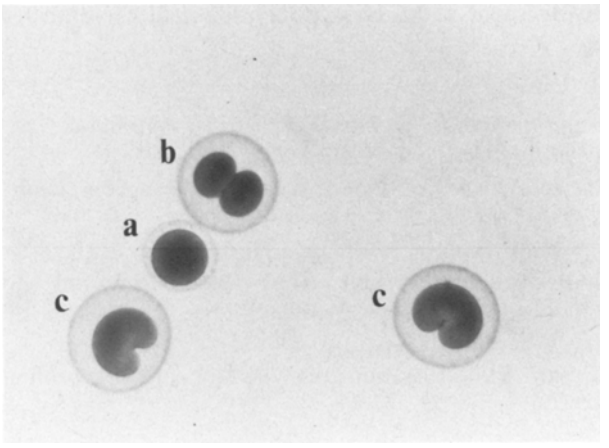

Figure 2. Photomicrograph of *H. roretzi* eggs treated with 0.05 mM Z-Pro-prolinal. The picture was taken at 105 min after insemination. *a* An unfertilized egg; *b* an egg at the two cell stage; *c* an egg with the furrow.

The similarity between the inhibitory effects of the protease inhibitors and of colchicine was also demonstrated using light microscopy; Z-Pro-prolinal induced transient formation of a furrow in the inseminated egg (fig. 2). The furrow disappeared at the time (2 h after insemination) when the inseminated egg without inhibitor treatment underwent a division into two cells. Therefore, the cleavage of the egg with the inhibitor treatment appeared to be suppressed when observed after 2 h. Colchicine also induced furrow formation in inseminated eggs.

As a preliminary study, we examined whether the time-dependent inhibition detected with prolyl endopeptidase inhibitors and colchicine in *H. roretzi* (Pleurogona) could also be demonstrated in the other order (Enterogona) of ascidians, for example in *Ciona savignyu,* in which chorion elevation was not observed and the first cleavage occurred at 2 h after insemination at 14 °C. It was found that the protease inhibitors and colchicine inhibited the first cleavage of *C. savignyu* eggs in a similar manner to that seen in *H. roretzi* eggs. Thus, the prolyl endopeptidase may function in a colchicine-sensitive process of fertilization in the ascidians.

In *H. roretzi,* oocytes arrested at the metaphase of the first meiosis are released at spawning time and resume their meiosis upon fertilization. The release of the first polar body occurs at 30 min after insemination, followed by the release of the second one at 45 min. The egg nucleus that has completed meiosis transforms into a female pronucleus and then fuses with the male pronucleus. After production of a single zygote nucleus, the first cell cleavage takes place. Taking account of the similarity in the timing of the action of the prolyl endopeptidase inhibitors and colchicine, it can be thought that the enzyme may function in a process between chorion elevation and

the first cell cleavage, probably sensitive to colchicine and also situated between pronucleus formation and the first cell cleavage. Further studies are necessary to define the precise role of prolyl endopeptidase in fertilization, and to search for the natural target materials (substrates) for the enzyme.

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Presence-absence cycles of the mother and not light-darkness are the zeitgeber for the circadian rhythm of newborn mice

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Summary. The relative roles of conflicting zeitgebers [presence/absence (PA) cycles versus light/dark (LD) cycles] on entrainment of circadian rhythm of locomotor activity were tested in pups of the night active mouse *Mus booduga*. During the early days of the pups' life the PA cycles of the mother acted as a zeitgeber and entrained their activity rhythm, even though the LD cycles were available. Entrainment by LD cycles took place only when the pups' eyes opened and probably became functional.
Key words. Maternal entrainment; circadian; zeitgeber; activity rhythm; *Mus booduga*.

In previous studies we demonstrated that presence/absence (PA) cycles of the mother mouse act as a zeitgeber and entrain the circadian rhythm of locomotor activity in *Mus booduga* pups under continuous darkness or continuous dim light [2–4]. Pups of this species, which is altricial and burrow-dwelling, rely behaviourally on the mother and take her presence as day and absence as night. Such mother/infant interactions probably help the pups to maintain the phase relationship of the prenatally set clock [5, 6] to that of the environment. In nature, the pups open their eyes on days 12–14 [2, 7], are weaned on around days 22–24 and start independent life at 30–35 days [8, 9]. It is well known that light/dark (LD) cycles are powerful and nearly universal zeitgebers of several circadian systems [10, 11]. In mammals the photic entrainment of the circadian clock occurs only via the retina during development [10–13]. Thus it appears that behavioural maternal entrainment could be expected to persist for a few days, until the time retina-mediated pathways become functional so that the developing clock can be synchronized directly to the physical zeitgebers such as environmental LD cycles. It is possible that during the early days of the pups' life a transition occurs in their circadian activity rhythm from a state of entrainment by PA cycles to a new state of entrainment again, this time by LD cycles. I have tested this hypothesis in the night active mouse *M. booduga*.

Materials and methods. Pregnant *M. booduga* were captured in the fields around the university campus. They were maintained in LD cycles (L: 06.00–18.00 h and D: 18.00–06.00 h) and produced litters of 2–8 pups each. The light intensity available to the animals was about 8–10 lx (Light source: incandescent bulbs – Philips, India). The animals for these experiments were a total of 8 mothers and 16 pups. The day of birth was designated as day 0. Starting on day 5 two pups of either sex were selected from each litter, named A and B and placed separately in plastic boxes of 21 × 15 × 13 cm. The mothers were presented for 12-h periods alternately to the pups, thereby creating PA cycles of 12:12 h. Thus the A pups were berthed with their mothers between 12.00–24.00 h and B pups between 24.00–12.00 h. Therefore the pups experienced behavioural and physical zeitgebers displaced relative to each other by 90° (figs 1 and 2).