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Zinc increases the longevity of unfertilized sea urchin eggs

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Summary. We have found that Zn^{2+} prevented lysis of unfertilized sea urchin eggs, and the eggs retained the ability to form fertilization membranes and to divide. SDS polyacrylamide gel electrophoresis showed that proteolysis of several proteins accompanied egg lysis, but Zn^{2+} inhibited this proteolysis. Therefore, Zn^{2+} blocks protease activity directly or indirectly and thereby prolongs the longevity of unfertilized sea urchin eggs.
Key words. Longevity; sea urchin eggs; zinc; cell death; protease.

Unfertilized sea urchin eggs have a limited life span. When unfertilized eggs are shed into seawater, they gradually lose the ability to form a fertilization membrane, and undergo disintegration by cytolysis [1, 2]. Many methods had been used to increase their longevity [1-4], but the factor(s) controlling the life span of sea urchin eggs are not known. Recently, we have found that Zn^{2+} increases the longevity of unfertilized eggs and prevents proteolysis of some egg proteins. We regard the lysis of unfertilized eggs as a kind of programmed cell death. Programmed cell death occurs in diverse organisms, but the mechanisms underlying it are unknown [5, 6]. Here we report that Zn^{2+} is useful for the study of the mechanism controlling longevity in unfertilized sea urchin eggs.

Materials and methods

Gametes. Anthocidaris crassispina were collected from the local coast of Toyama Bay and kept in running seawater aquaria at 25 °C. Shedding of gametes was induced by injecting 0.5 M KCl into the coelomic cavity. Eggs were shed into filtered natural seawater (FNSW) and washed three times with FNSW. Sperm was collected 'dry' in plastic dishes and stored at 4 °C until use.

Treatments. Unfertilized eggs were transferred into FNSW containing various concentrations of Zn^{2+} immediately after collecting and washing. In some experiments, eggs were first homogenized in FNSW by 15 strokes of a Teflon homogenizer. All eggs and egg-homogenates were kept in 50-ml plastic tubes rotated at 5 rounds/min (Taitec Rotator RT-50, Taiyo Service Center Co., Ltd., Tokyo) at 25–27 °C (the temperature of the seawater in the spawning season of this sea urchin). Densitiy of eggs was adjusted to about 5% (v/v). A stock solution of $ZnCl_2$ (100 mM) in deionized water was diluted with FNSW to appropriate concentrations.

Measurement of longevity. Aliquots of experimental eggs were taken at suitable time intervals. Some aliquots were fixed immediately in 1% glutaraldehyde in FNSW for analysis of lysed eggs, and some were washed three times with FNSW and inseminated to assay their ability to form a fertilization membrane and to divide. Microscopic observations were made with an inverted microscope (Olympus CK-2).

Gel electrophoresis. Sample eggs were washed three times with FNSW and homogenized in FNSW with a Teflon homogenizer. Protein concentrations of homogenates were measured by the method of Bradford [7] with bovine serum albumin as the standard, and were subjected to SDS polyacrylamide gel electrophoresis according to the method of Laemmli [8]. Gels were stained with silver [9].

Results

When unfertilized eggs of the sea urchin *Anthocidaris crassispina* were kept in FNSW at 25–27 °C, they gradually disintegrated (fig. 1). The cytoplasm became heterogeneous or transparent, the surface often blebbed, and eventually the eggs lysed. Although the percentage of disintegrated eggs varied in different batches, more than 90% of the eggs lysed 20–24 h after shedding. However, disintegration of eggs was largely prevented when eggs were treated with 1 mM Zn^{2+}; only about 10% of eggs lysed during the same period (fig. 1). This protective effect of Zn^{2+} was observed at concentrations over 0.05 mM, but 1 mM Zn^{2+} showed the most stable and the strongest effect. To exclude the possibility that eggs lysis was caused by bacterial digestion, in some experiments eggs were washed with fresh FNSW at each measurement point. Nevertheless, egg longevity was not increased by this treatment.

Unlysed eggs in Zn^{2+}-FNSW retained their ability to form a fertilization membrane (fig. 2) and to divide (fig. 3). However, the percentage of eggs with a fertilization membrane decreased with duration of Zn^{2+}-treatment, and development of embryos stopped at around the morula stage after 24 h of Zn^{2+}-treatment. Ability to form a fertilization membrane was always lost faster than the ability to divide.

Effects of other divalent cations (0.1–1 mM) were also examined. Cd^{2+} had the same effects on unfertilized eggs as Zn^{2+}, but Ca^{2+}, Mg^{2+}, Cu^{2+} and Mn^{2+} had no effect. When eggs were treated with Hg^{2+}, disintegration of eggs was blocked, but the cytoplasm became transparent and the ability to form a fertilization membrane and to divide were lost more rapidly than in control eggs.

Proteins from whole eggs were resolved by electrophoresis on SDS-gels (fig. 4). Eggs kept in FNSW displayed three new major bands at 160 kDa, 110 kDa, 64 kDa, 12 h after shedding, and two additional bands at 105 kDa and 25 kDa appeared after 24 h. Several minor protein bands also appeared in samples from untreated eggs. On the other hand, eggs kept in FNSW containing 1 mM Zn^{2+} showed no protein changes on gels. When eggs were homogenized first and kept in FNSW or FNSW containing 1 mM Zn^{2+}, a degradation pattern the same as that in whole eggs was observed.

Discussion

The result presented here show that Zn^{2+} prolongs the life span of unfertilized eggs of the sea urchin *Anthocidaris crassispina*. The same effects on unfertilized eggs were also observed in two other sea urchins *Hemicentrotus pulcherrimus* and *Pseudocentrotus depressus* (data not shown). It is well known that the actions of

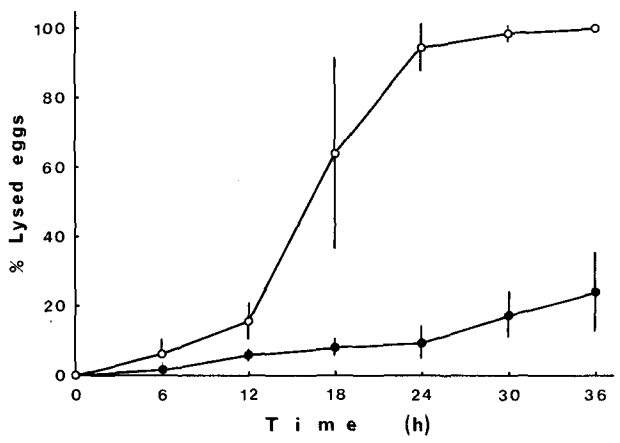

Figure 1. Effect of Zn^{2+} on lysis of unfertilized eggs of the sea urchin, *Anthrocidaris crassispina*. Immediately after collecting and washing, eggs were transferred into filtered natural seawater (FNSW) or FNSW containing 1 mM Zn^{2+}. These eggs were kept in plastic tubes which were rotated (5 rounds/min) at 25–27 °C. Lysed eggs were counted with an inverted microscope. Data represent mean ± SD of four different experiments (a measurement of 100–300 eggs). ○, Eggs in FNSW; ●, eggs in FNSW containing 1 mM Zn^{2+}.

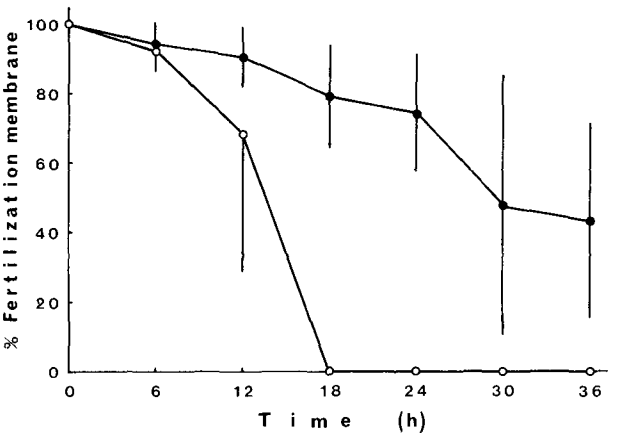

Figure 2. Effect of Zn^{2+} on loss of ability to form a fertilization membrane. Aliquots of experimental eggs were taken at each data point, washed three times with FNSW, then inseminated. The percentage of eggs with a fertilization membrane was measured at 10 min after insemination with an inverted microscope. Data represent mean ± SD of four different experiments. ○, Eggs in FNSW; ●, eggs in FNSW containing 1 mM Zn^{2+}.

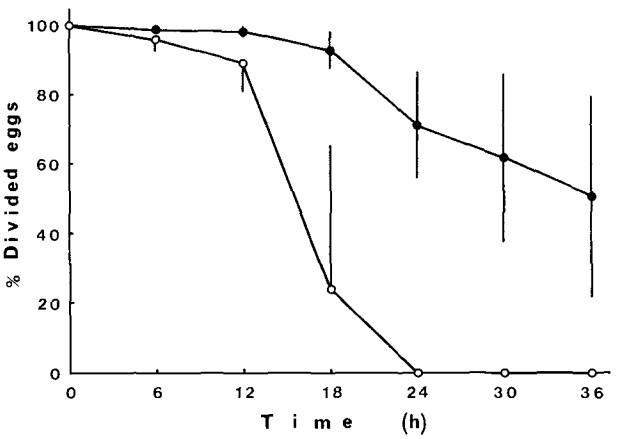

Figure 3. Effect of Zn^{2+} on loss of ability to divide. The percentage of divided eggs was measured at 4–6 h after insemination as described in fig. 2. Data represent mean ± SD of four different experiments. ○, Eggs in FNSW; ●, eggs in FNSW containing 1 mM Zn^{2+}.

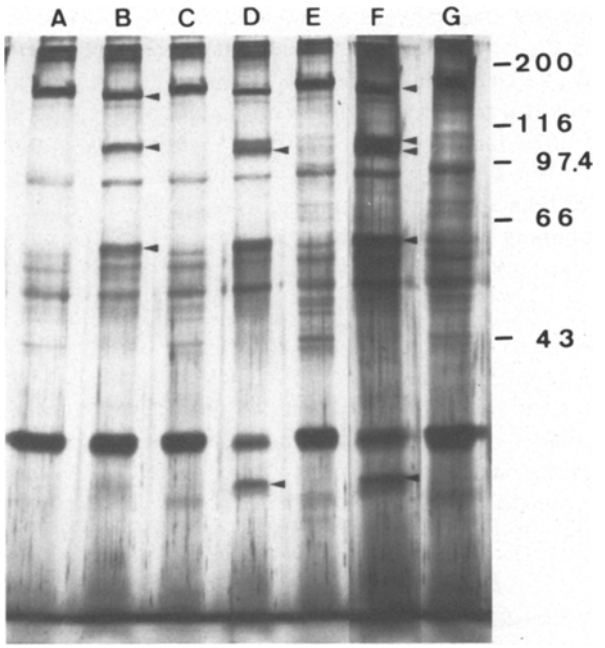

Figure 4. SDS-polyacrylamide gel electrophoresis of whole egg-homogenates. A 7–10% separation gel and a 3% stacking gel were used. Eggs were kept for 0 h (A), 12 h (B and C), and 24 h (D and E) and homogenized. A, B and D, homogenates of eggs kept in FNSW; C and E, homogenates of eggs kept in FNSW containing 1 mM Zn^{2+}. In the same batch of eggs, eggs were homogenized at first and kept for 24 h in FNSW (F) or FNSW containing 1 mM Zn^{2+} (G). Arrowheads show new bands. Molecular mass standards are indicated on the right in kilodaltons. Gels were stained with silver.

Zn^{2+} on sea urchin eggs and embryos include: a) induction on animalization [10] and b) inhibition of formation of the fertilization membrane [11–14]. We now can add another action of Zn^{2+} on sea urchin eggs.

Many studies have attempted to maintain unfertilized sea urchin eggs for long periods [1–4]. However, little is known about the factors controlling egg longevity. Aketa [3] reported that adenosine prevented loss of fertilizability and he postulated that adenosine acts on the egg surface and somehow raises ATP levels [4]. On the other hand, our results show that Zn^{2+} inhibits proteolysis, and increases the life span of eggs. We do not know whether Zn^{2+}-inhibition of proteolysis is a direct or indirect effect. Preliminary experiments indicate that serine protease(s), such as chymotrypsin, are involved in the proteolysis. Moreover, we suppose that Zn^{2+} acts in the cytoplasm because when egg-homogenates were kept in FNSW with or without Zn^{2+}, they showed the same degradation pattern in SDS-PAGE as was seen in whole eggs treated with and without Zn^{2+}. Zinc, cadmium and mercury belong to group 2b of the periodic table, so that they might be expected to have the same effect on egg lysis. When unfertilized eggs were kept in FNSW, the cytoplasm became heterogeneous or transparent, the egg surface blebbed, and finally the egg lysed. From these observations we propose a minimal program for egg death: (1) disturbance of cortical organization and focal weakening of egg surface, (2) changing of membrane permeability, (3) rupture of cytoplasm, (4) activation of internal proteases, (5) disintegration of eggs. Steps (1) and (2) are not necessary for step (4), because whole eggs and egg-homogenates in FNSW have the same degradation pattern in SDS-PAGE. Future experiments on the longevity of unfertilized sea urchin eggs may provide important clues for understanding the as yet unknown mechanism of programmed cell death.

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