ZINC INDUCES OOCYTE MATURATION IN CHINESE TOAD (*Bufo bufo gargarizans*)

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ABSTRACT

Progesterone can induce maturation (as indicated by germinal vesicle breakdown, GVBD) of amphibian oocytes *in vitro*. GVBD was induced by zinc alone in place of progesterone, both in Chinese toad (*Bufo bufo gargarizans*) and in *Xenopus*. In the present study, 500 μ M ZnCl₂ was found to induce complete GVBD only when applied for 5 h to cultured oocytes from Chinese toad. In contrast, oocytes required only a short period (5 min) of exposure to progesterone for GVBD to occur. Cytoplasm from oocytes matured in the presence of zinc induced GVBD when microinjected into immature oocytes. Such results demonstrate that completion of GVBD induced by zinc involved release of probable 'maturation promoting factor'. Furthermore, zinc itself was not able to induce GVBD when injected into the oocytes. However, external application with progesterone or zinc induced GVBD. We report here for the first time that zinc acts primarily at the superficial plasma membrane sites, since externally applied zinc induced GVBD. Zinc induced maturation presents several characteristics common to progesterone-induced maturation.

Zinc is an intracellular signaling molecule that controls a wide array of cellular processes (12). A role for zinc has been suggested in oocyte maturation (as indicated by germinal vesicle breakdown, GVBD) of *Xenopus laevis* induced by progesterone (1, 2, 13, 14). When oocytes were treated with a zinc-chelating agent such as *o*-phenanthroline, progesterone-induced GVBD was prevented (14). Thus, intracellular zinc, which is present in *Xenopus* oocytes (9, 11), is thought to be involved in oocyte maturation. In contrast, oocyte maturation occurred after stimulation with zinc alone in the absence of progesterone (1, 2, 13, 14). In addition, when oocytes were loaded with an inhibitor of protein synthesis, such as cycloheximide, zinc-induced GVBD was prevented (14). Thus, intracellular zinc-transients appear to be involved in the progression of GVBD. Such results prompted us to

investigate a possible role for zinc as a relay between zinc-binding and maturation.

During maturation, *Xenopus* oocytes, which induce maturation when injected into unstimulated oocytes, contain maturation promoting factor (MPF) in the cytoplasm (6, 7). MPF is composed of a p34^{cdc2} protein kinase and its regulatory cyclin B1 subunit and is required for GVBD (3, 6). In this study, we investigated using oocytes from Chinese toad (*Bufo bufo gargarizans*) whether or not zinc by itself can induce GVBD and whether or not cytoplasm from zinc-treated matured oocytes can induce GVBD in recipient oocytes in the absence of hormone.

MATERIALS AND METHODS

Animal

Adult female *Bufo bufo gargarizans* was obtained in China (Jiangsu province) in December, then kept in hibernation at 4°C until used.

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Fig. 1 Effect of ZnCl_2 -treatment on toad oocytes. Oocytes from a particular animal were incubated at $19 \pm 1^\circ\text{C}$ for 20 h in Ringer solution containing different concentrations (62.5, 125, 250 and $500 \mu\text{M}$, respectively) of ZnCl_2 and then GVBD was assessed. Each point represents the mean $\pm$ SE of %GVBD for each assay from four animals.

Chemicals

ZnCl_2 was purchased from Sigma Chemical (U.S.A.). Progesterone and other agents were of analytical reagent grade from the Shanghai Factory of Biochemistry (China).

Preparation of Oocytes

Animals were sacrificed, and their ovaries were removed and intact, fully-grown oocytes were separated from the ovarian follicular envelope with forceps under a dissecting microscope.

In vitro Oocyte Culture

Usually, all incubations were carried out using 10 or 20 oocytes for each group in 15 or 20 ml of Ringer solution (101.7 mM NaCl, 1 mM KCl, 0.9 mM CaCl_2 and 1.2 mM NaHCO_3 ; adjusted to pH 7.4 with HCl) containing $1 \mu\text{M}$ progesterone (1 mM stock solution in ethanol) or $500 \mu\text{M}$ ZnCl_2 (500 mM stock solution) at $19 \pm 1^\circ\text{C}$ for 20 h. Oocytes were then fixed in 5% trichloroacetic acid and examined under a dissecting microscope. The percentage of oocytes that had undergone GVBD was determined.

Assay of MPF

Donor oocytes were incubated in Ringer solution

Fig. 2 Time course of GVBD after initiation of ZnCl_2 -treatment. Oocytes from a particular animal were incubated at $19 \pm 1^\circ\text{C}$ in the continuous presence of $500 \mu\text{M}$ ZnCl_2 (●) or $1 \mu\text{M}$ progesterone (○) and fixed at regular intervals. Then, GVBD was assessed. Each point represents the mean $\pm$ SE of %GVBD for each assay from four animals.

containing $500 \mu\text{M}$ ZnCl_2 or $1 \mu\text{M}$ progesterone at $19 \pm 1^\circ\text{C}$ for 20 h. Usually, cytoplasm taken from donor oocytes was microinjected into recipient oocytes at a volume of about 50 nl per oocyte. After injection, recipient oocytes were washed five times with Ringer solution and stored in the same solution at $19 \pm 1^\circ\text{C}$ for 20 h, then the percentage of GVBD was checked. Microinjection proceeded as described elsewhere (4).

RESULTS

Induction of GVBD by Zinc

Fig. 1 shows the effect of increasing doses (62.5– $500 \mu\text{M}$) of ZnCl_2 on GVBD. The percentage of oocytes that underwent GVBD was 27.5% at $62.5 \mu\text{M}$ ZnCl_2 , and 72.4% at $125 \mu\text{M}$ ZnCl_2 . GVBD (100%) was maximal at a dose of 250– $500 \mu\text{M}$ ZnCl_2 . These results showed that zinc mimics progesterone action in inducing GVBD as in *Xenopus* (1, 2, 13, 14).

Comparison of the Time Course of Zinc- and Progesterone-Induced GVBD

We compared the time course of GVBD after exposing oocytes from the same animal to zinc and progesterone (Fig. 2). At $500 \mu\text{M}$ ZnCl_2 , GVBD began within 6 h and finished after 7–8 h. This time course was similar to that with $1 \mu\text{M}$ progesterone suggesting that zinc- and progesterone-

Fig. 3 Effect of ZnCl_2 exposure on oocytes. Oocytes from a particular animal were placed at $19 \pm 1^\circ\text{C}$ in Ringer solution containing $500 \mu\text{M}$ ZnCl_2 (●) or $1 \mu\text{M}$ progesterone (○). After different periods (5 and 10 min, 1, 2, 3, 4, 5 and 6 h with ZnCl_2 or 5 and 10 min with progesterone), oocytes were washed and cultured in agent-free Ringer solution for a final 20 h. Oocytes were fixed and then GVBD was assessed. Each point represents the mean $\pm$ SE of %GVBD for each assay from four animals.

induced maturation are stimulated by a similar sequence of events.

Comparison of the Length of Exposure to Zinc and Progesterone of Oocytes Required for GVBD Induction

Fig. 3 shows that the incidence (100%) of GVBD was highest when oocytes were exposed to progesterone for 5 or 10 min, then washed and incubated for a further 20 h in progesterone-free medium. However, oocytes treated for 5 min, 10 min, 1 h or 2 h with zinc remained immature whereas those exposed for 3 h or more underwent GVBD. The frequency of induced GVBD was 3% at 2 h, 37% at 3 h, 65% at 4 h and 93% at 5 h, respectively. These results showed that the frequency of zinc-induced GVBD is proportional to the duration of exposure to zinc. Thus, it appears that oocytes require a much longer exposure to zinc than to progesterone for GVBD induction.

Appearance of MPF Activity in Cytoplasm from Zinc-Treated Oocytes

Cytoplasm (50 nl), taken from $500 \mu\text{M}$ ZnCl_2 -treated oocytes undergoing GVBD, was capable of

Table 1 Injection of Cytoplasm into Immature Oocytes

Source of cytoplasm injected	%GVBD
ZnCl_2 -treated oocytes ^a	81.3 ± 8.4 (n = 40)
Progesterone-treated oocytes ^b	89.0 ± 6.7 (n = 32)
Control ^c	0 (n = 42)

GVBD in recipient oocytes was examined 20 h later after the injection of cytoplasm (50 nl) from donor oocytes treated for 20 h with $500 \mu\text{M}$ ZnCl_2 ^a or $1 \mu\text{M}$ progesterone^b. In addition, ZnCl_2 (35 nl as 500 – $2,500 \mu\text{M}$ solution) injected as a control^c was assayed for GVBD. Values are the mean $\pm$ SE of %GVBD in recipient oocytes for each assay. Number (n) in parentheses is the total number of oocytes used from four animals.

inducing GVBD after 20 h when injected into untreated recipient oocytes (Table 1). In addition, 35 nl of $500 \mu\text{M}$ ZnCl_2 solution had no apparent effect when injected into untreated and recipient oocytes. Moreover, injection of cytoplasm from $1 \mu\text{M}$ progesterone-treated oocytes induced GVBD as expected (7). Based on these results, we concluded that zinc was effective in inducing GVBD when applied to the outside of oocytes.

Rescue Effect of Progesterone or Zinc on GVBD of Recipient Oocytes Injected with Zinc

We examined whether GVBD was induced when recipient oocytes injected with zinc were incubated with progesterone or zinc, since high doses of zinc, compared to those listed in Table 1, did not induce maturation when injected into the oocytes (Table 2). Injection of 35 nl of 2, 5 and 15 mM ZnCl_2 solution did not induce GVBD, however, the activities of recipient oocytes were rescued following incubation with progesterone. Incubation of oocytes injected with 35 nl of 15 mM ZnCl_2 solution, with $500 \mu\text{M}$ ZnCl_2 also induced maturational activity. The induction of GVBD by zinc was dependent on the presence of zinc in the external medium. Recipient oocytes without activity were neither killed nor inhibited by high zinc concentrations. Thus, zinc was expected to act primarily at the superficial plasma membrane sites.

DISCUSSION

We have shown that oocytes from the Chinese toad (*Bufo bufo gargarizans*) could be induced to undergo GVBD following exposure to zinc, similar to oocytes from *Xenopus laevis* (14). Further, zinc did

Table 2 Rescue Effect of External Application of Progesterone or ZnCl_2 on Oocytes Injected with Different Doses of ZnCl_2

ZnCl_2 (mM) injected	Addition to the external medium	%GVBD	
2	Vehicle ^a	0	(n = 38)
	Progesterone ^b	95.0 $\pm$ 5.0	(n = 25)
5	Vehicle ^a	0	(n = 37)
	Progesterone ^b	100	(n = 25)
15	Vehicle ^a	0	(n = 37)
	Progesterone ^b	100	(n = 24)
	ZnCl_2^c	95.0 $\pm$ 5.0	(n = 23)

Twenty hours after 2, 5 or 15 mM ZnCl_2 -injection (35 nl) into oocytes, their recipient oocytes were treated with vehicle (control)^a, 1 μM progesterone^b or 500 μM ZnCl_2^c , and scored 20 h later for GVBD. Values are the mean $\pm$ SE of %GVBD in recipient oocytes for each assay. Number (n) in parentheses is the total number of oocytes used from four animals.

not induce GVBD when injected into the oocyte, and was effective only when applied to the outer surface. Accordingly, it has been hypothesized that zinc primarily interacts with components located on or near the outer surface of oocytes. Thus, it is likely that external application of zinc caused GVBD through the formation of MPF.

Oocyte maturation induced by zinc was similar to that induced by progesterone in several ways. The transfer of cytoplasm containing MPF from zinc-matured oocytes to immature oocytes not exposed to hormones, resulted in GVBD (Table 1). However, injection of zinc alone was not able to induce GVBD, at any dose (500 μM –15 mM) (Tables 1 and 2). These doses can produce an internal additive concentration as high as 18 to 525 μM ; the endogenous concentration of zinc is 1 mM (11), assuming that the total volume of an oocyte is 1 μl and the zinc is uniformly distributed within the oocyte. However, 20 h later, when oocytes were treated with progesterone or zinc, GVBD was induced. These results suggest that zinc acts primarily not from within the cytoplasm, but rather through a site accessible from the exterior. That zinc induced maturation makes it likely that it is involved in the initiation of maturation. Progesterone as a physiological inducer is known to cause GVBD in *Xenopus* oocytes through the oocyte surface and the formation of cytoplasmic MPF (6, 7). Zinc and progesterone likely induce GVBD in toad oocytes through a similar mechanism. However, some difference was noted. When oocytes were treated with 500 μM ZnCl_2 alone, a 5-h exposure

to zinc was necessary for GVBD completion, compared with a 5-min exposure to progesterone. Thus, it appears that the movement of zinc is highly regulated, or the level of zinc needs to be sustained, during the period of maturation.

In frog oocytes studied to date, the physiological stimulus for maturation and ovulation (or shedding) produced changes in ion permeability, ion transport and/or ion binding by plasma membrane (4, 10, 15). Here, the initiation of *in vivo* maturation, which requires the presence of zinc in the external medium, appears to depend on an influx of ions containing zinc from the medium following progesterone stimulation. In addition, it is known that *Xenopus* oocytes take up zinc through oogenesis until fully-grown, when the concentration is 1 mM (11). However, little is known regarding the entry of zinc into cells including frog oocytes. Mammalian intestine is thought to take up zinc both by passive diffusion and by carrier-mediated processes as yet uncharacterized. Similar mechanisms in frog oocytes could involve receptor-mediated endocytosis of vitellogenin as a plasma zinc-binding protein (9). Once internalized, vitellogenin is cleaved into two lipovitellin subunits and phosvitin, and the zinc of vitellogenin associates with the lipovitellin domains.

It may well be that zinc has functions in other proteins and enzymes (8, 12). Zinc may be an intracellular messenger linked to the regulation of oocyte maturation induced by progesterone as shown in the growth and differentiation of mammalian cells (12). It is of interest that the degrada-

tion of clam cyclin, a component of MPF, may be regulated *in vitro* by zinc (3, 5). In the presence of 250 μ M and 1 mM ZnCl_2 , cyclin destruction was inhibited, whereas 125 μ M ZnCl_2 delayed destruction. Whether zinc affects physiological changes in ion permeability, ion transport and/or ion binding by plasma membrane of frog oocytes, and how it induces oocyte maturation, is not clear. More detailed studies on the zinc-dependent systems underlying the biological process of frog oocyte maturation are needed.

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