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REINITIATION OF MEIOSIS IN STARFISH OOCYTES REQUIRES AN INCREASE IN NUCLEAR CA^{2+}

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Here we report that an increase in nuclear Ca^{2+} is hormonally regulated and is required for reinitiation of meiosis in starfish oocytes. We have shown in *Asterina pectinifera* that a Ca^{2+} transient and a baseline increase of Ca^{2+} occur both in the cytoplasm and in the nucleus. These increases are in response to 1-methyladenine (1-MA), the hormone responsible for the induction of meiotic maturation. The Ca^{2+} transients are not necessary for the reinitiation of meiosis. However, blocking the baseline increase in nuclear Ca^{2+} blocks both the nuclear envelope breakdown (NEBD) and the continuation of meiosis.

Changes in cytosolic Ca^{2+} control a variety of cellular processes (1-4). The increase in cytosolic Ca^{2+} preceding nuclear envelope breakdown (NEBD) is essential for entry into mitosis in sea urchin embryos (5,6). Within the nucleus, Ca^{2+} plays an indirect role in regulation of nuclear functions (7). The pioneering work of Dalcq (8) and Pasteels (9) indicated the importance of Ca^{2+} in triggering meiosis reinitiation (maturation) in starfish oocytes. These oocytes undergo final maturation (10, 11) in response to 1-methyladenine (1-MA), which acts directly on the plasma membrane (12) and renders the oocyte fertilizable (13-15). Using the calcium-sensitive luminescent protein aequorin, it was shown that the binding of 1-MA to cell surface receptors triggers a transient increase in intracellular Ca^{2+} in starfish oocytes (16,17). However, other investigators found no change in free calcium during 1-MA induced maturation of aequorin-injected oocytes (18). Because buffering cytoplasmic Ca^{2+} by injecting EGTA or BAPTA did not inhibit meiosis (17,19), it was suggested that the Ca^{2+} transient is not necessary for maturation.

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Materials and Methods

Starfish of *A. pectinifera* were collected from the Mutsu Bay near Asamushi Marine Biological Station (Japan) at the beginning of September and kept in running sea water. Fully grown oocytes with germinal vesicles were isolated from ovaries and washed several times in filtered sea water. (The terms germinal vesicle (GV) and nucleus are used interchangeably).

Microinjection procedures: The germinal vesicle and the cytoplasm of the oocytes were microinjected with a solution of 5 mM fura-2 in 100 mM potassium aspartate, 10 mM Hepes (pH 7.0). Injections were performed with a micromanipulator (Leitz) using capillary pipettes according to published methods (20). Fura-2 was injected at 23°C. The injected volumes were adjusted so that final concentrations of fura-2 in the cytoplasm and the nucleus were ~ 50 μM. After injecting fura-2, the external solution was replaced with fresh sea water and the fura-loaded oocytes transferred to the measurement chamber at 18°C.

Calcium measurement: Calcium concentrations were determined by continuous observation using an inverted microscope (Olympus IMT-2) equipped with an epifluorescence monitor (Olympus IMT-RFL) and a computer for the measurement of Ca^{2+} (Olympus OSP-3). Free calcium levels were determined from ratio measurements of the fluorescence of fura-2 values from excitation at 340 nm and 385 nm within a circular area 50 μm in diameter. Extrusion of GV was achieved by manual dissection at various times after injection. The solution used for injection of blocking buffer BAPTA (100 mM BAPTA; 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, Dojin) was added to 100 mM potassium aspartate, 10 mM Hepes (pH 7.0) and injected in the nucleus; 200 mM BAPTA was injected in the cytoplasm.

Transmission electron microscopy: Samples were fixed in 1% glutaraldehyde in sea water for 1 hour and then post-fixed in 1% OsO_4 in sea water for 1 hour. The samples were dehydrated in a graded alcohol series and embedded in Epon 812. Sections were stained with 2% uranyl acetate and 0.2% lead citrate and examined with a Philips 400 transmission electron microscope.

Results and Discussion

Starfish oocytes (*Asterina pectinifera)* arrested in late prophase of the first maturation division are characterized by a large (60 μm in diameter) nucleus. Just before spawning, immature oocytes resume meiotic division as indicated by nuclear envelope breakdown (NEBD). We found that fura-2 injected into either the GV, or the cytoplasm, of immature oocytes remained in that compartment. Fura-2 (5 mM) injected into the nucleus remained there for at least 1 hour whether the nucleus remained in the cell (Fig. 1A) or was dissected out and placed in sea water. Likewise, fura-2 injected into the cytoplasm remained there; after 1 hour the isolated GV did not have fluorescence (Fig. 1B). Additional evidence that fura-2 injected into the cytoplasm does not enter the nucleus until NEBD comes from fura-2 light emission at the time of NEBD (20 min after 1-MA addition; Fig. 1C).

We found that following 1-MA treatment there are, in fact, three Ca^{2+} transients, two in the cytoplasm (cyto 1 and cyto 2), and one in the nucleus (Fig. 2). In all oocytes in which both cytoplasm and nucleus were injected with fura-2, near-simultaneous measurement of the Ca^{2+}

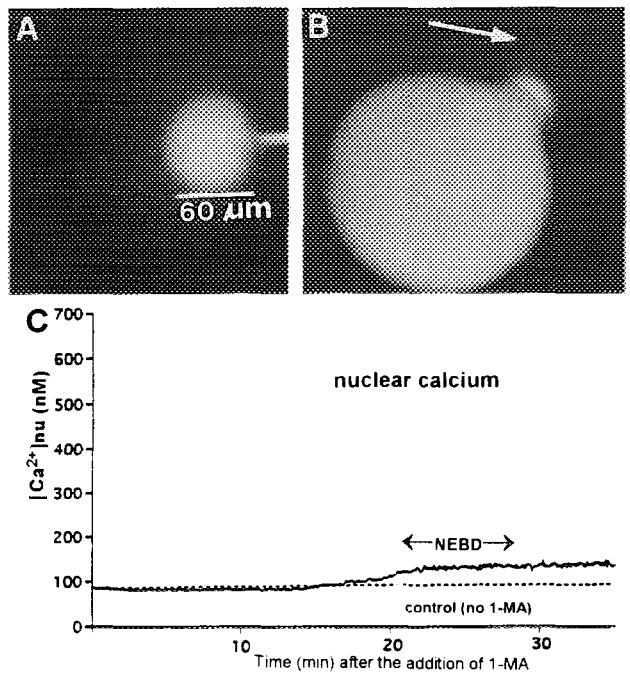

Figure 1. Oocytes of *Asterina pectinifera* with fura-2 injected into the nucleus as seen with ultraviolet light. The fluorescent compound is retained for at least 1 hour (**A**). Oocytes with fura-2 injected into the cytoplasm. The fluorophore remains in the cytoplasm for at least 1 hour and does not accumulate in the germinal vesicle (arrow), which was manually extruded from the cell 1 hour after cytoplasmic injection (**B**). Nuclear calcium monitored continuously after cytoplasmic injection of fura-2 and after addition of 1 μM of the hormone 1-MA. Only at the time of nuclear envelope breakdown (NEBD) is it possible to observe an increase in baseline nuclear Ca^{2+}. The Ca^{2+} concentration was calculated by ratiometric measurement every 5 seconds (**C**).

in each compartment showed that the nuclear peak always followed the first cytoplasmic Ca^{2+} peak (cyto 1) by about 20 seconds (Fig. 2C).

The source of nuclear Ca^{2+} and the question of whether the three transients are causally related were investigated. Partial blockage of the first cytoplasmic Ca^{2+} transient increase (cyto 1) occurred upon injection of BAPTA into the cytoplasm (a Ca^{2+} chelator that functions better at physiological pH). BAPTA injected into the cytoplasm blocked both the cytoplasmic transient (cyto 1) (data not shown) and the nuclear transient (Fig. 3A). Nevertheless after injection of BAPTA into the cytoplasm, both the cytoplasm and the nucleus (Fig. 3A) showed a gradual increase in baseline Ca^{2+} over the 40 minutes period after addition of 1-MA. Furthermore, 24 of 27 oocytes treated similarly underwent NEBD. These results suggest that 1-MA triggers an increase in cytosolic Ca^{2+} levels that is followed 20 seconds later by an

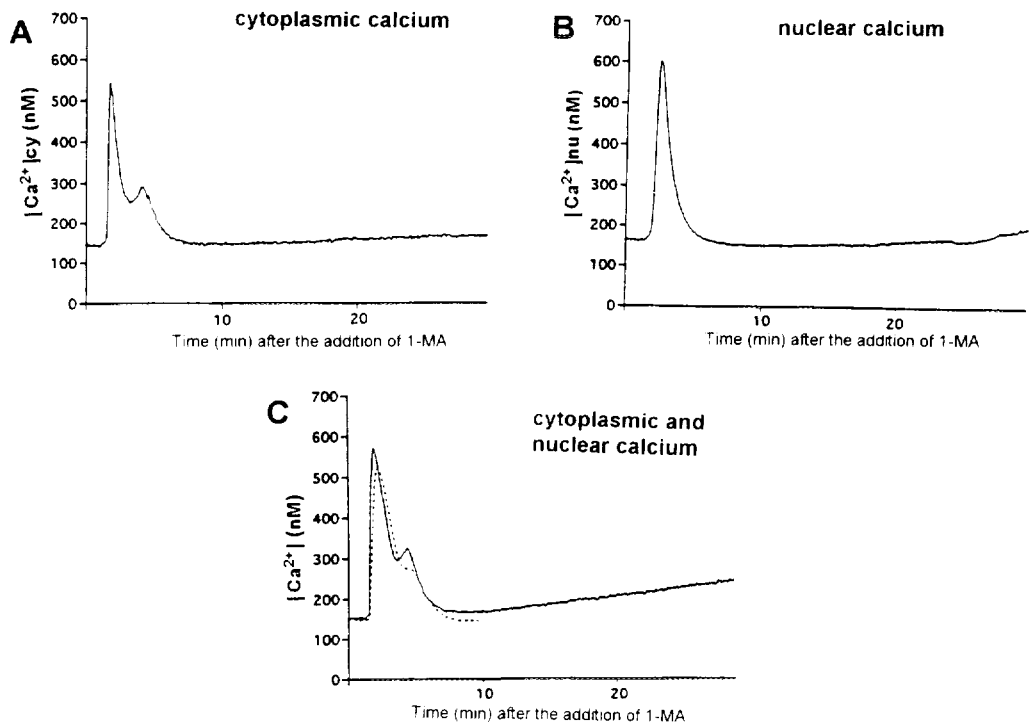

Figure 2. Responses of *A. pectinifera* oocytes to 1-MA following injection of fura-2 (50 μM): (**A**) into the cytoplasm, (**B**) into the nucleus, and (**C**) into both cytoplasm and nucleus. The diameter of the immature oocytes was ~185 μm and the germinal vesicle was ~60 μm in diameter. The oocytes were held between coverslips in the measuring chamber and slightly flattened to ~200 μm in diameter. This facilitated the measurement of fluorescence from the cytoplasm and the nucleus separately. In (**A**) the 50 μm measuring ring was placed over the cytoplasm, in (**B**) over the nucleus, in (**C**) alternately (every 5 sec) over the cytoplasm then the nucleus. (**A**) shows the typical cytoplasmic calcium transient peak followed 140 seconds later by a smaller transient. (**B**) shows that there is a Ca^{2+} transient of similar amplitude in the nucleus. (**C**) shows the nuclear Ca^{2+} transient (dotted line) that occurs 20 seconds after the first cytoplasmic transient (solid line).

increase in nuclear Ca^{2+}. The converse experiment is shown in Fig. 3B, in which we measured cytoplasmic Ca^{2+} increase after injecting 100 mM BAPTA into the nucleus. Blockage of the nuclear Ca^{2+} increase inhibited the second cytoplasmic Ca^{2+} peak (cyto 2) but left the first Ca^{2+} peak (cyto 1) unaffected. These results indicate that the second cytosolic transient depends on the nuclear transient. After injecting 100 mM BAPTA in the nucleus, NEBD and reinitiation of meiosis did not occur.

We believe that the normal sequence of events is as follows: 1-MA triggers an increase in cytoplasmic Ca^{2+} that has both a sharp transient (cyto 1 and cyto 2, Fig. 2) and a gradual baseline increase phase. In agreement with a previous report (19), we found that when we

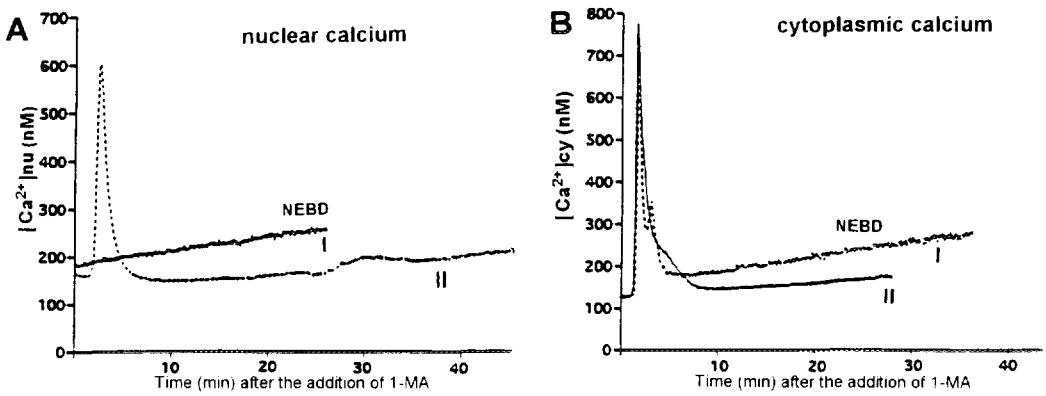

Figure 3. Responses of the germinal vesicles (GV) of starfish oocytes loaded with fura-2 after injection of BAPTA into the cytoplasm. (**A**) Note that even if BAPTA depresses the nuclear Ca^{2+} transient, an increase in baseline nuclear calcium occurs and NEBD is not affected. Final cytoplasmic concentration of BAPTA is ~2 mM. **I**: BAPTA in cytoplasm; **II**: Control. (**B**) Converse experiment to Fig. 3A but with BAPTA injected into the nucleus and fura-2 injected into the cytoplasm. Note that BAPTA injection suppresses the second cytoplasmic Ca^{2+} transient (cyto 2). Final nuclear concentration of BAPTA is ~0.5 mM. **I**: Control; **II**: BAPTA in nucleus.

inhibited the first cytoplasmic Ca^{2+} transient (cyto 1) by injecting BAPTA into the cytoplasm, NEBD occurred. However, our experiments show that injection of BAPTA into the cytoplasm does not prevent all Ca^{2+} elevation. NEBD is always accompanied by a measurable increase in the baseline Ca^{2+} in the cytoplasm and successively by an elevation in nuclear Ca^{2+} (Fig. 3A). We postulate that the increase in baseline nuclear Ca^{2+} is essential for the continuation of meiosis in starfish oocytes of *A. pectinifera* (Table 1).

Consistent with this hypothesis, after injection of 100 mM BAPTA into the nucleus, it was possible to measure only the first cytoplasmic Ca^{2+} transient (cyto 1); the second elevation

TABLE 1. Ratio of increase of intracellular Ca^{2+} of starfish oocytes following the addition of 1-MA

	NEBD	ratio $[Ca^{2+}]_{25\ min}/[Ca^{2+}]_{0min}$	
$[Ca^{2+}]_{cy}$	+	1.33 ± 0.29 (a)	n= 7
$[Ca^{2+}]_{cy}$	-	0.96 ± 0.01 (b)	n= 5
$[Ca^{2+}]_{nu}$	+	1.49 ± 0.22 (c)	n= 5

(a) Mean ± standard deviation. The ratio was calculated from the $[Ca^{2+}]_{cy}$ at 25 min after the addition of 1-MA to the 0 min $[Ca^{2+}]_{cy}$ (before the application of 1-MA). (b) NEBD was suppressed by injecting 200 mM BAPTA into cytoplasm before the application of 1-MA. (c) The ratio was calculated from the $[Ca^{2+}]_{nu}$ at 25 min after the addition of 1-MA to the 0 min $[Ca^{2+}]_{nu}$ (before the application of 1-MA).

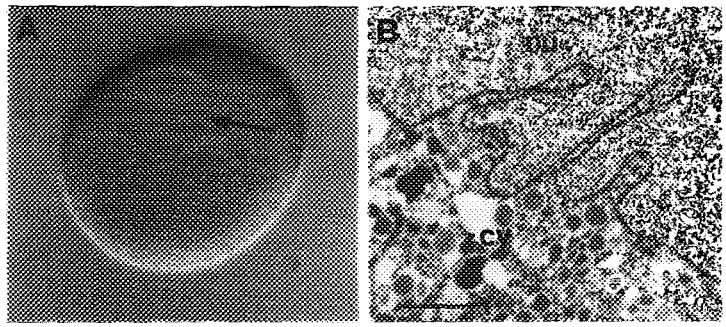

Figure 4. (**A**). Effect of nuclear BAPTA injection 1 hour after 1-MA addition. The intermixing of nucleoplasm and the cytoplasm is prevented (arrow) because (**B**) the nuclear envelope remained intact. nu=nucleus, cy=cytoplasm. Final nuclear concentration of BAPTA is ~2 mM. Bar, 5 μm.

of Ca^{2+} in the cytoplasm (cyto 2) was suppressed. As a result, the NEBD was inhibited, and the reinitiation of meiosis did not occur. These results indicate that 1-MA triggers the first cytoplasmic Ca^{2+} release (cyto 1) from the intracytosolic stores. Changes in the cytosolic Ca^{2+} levels causes the elevation of the nuclear Ca^{2+}, and inhibition of the nuclear Ca^{2+} transient suppressed the cyto 2 increase.

In *A. pectinifera* 30 minutes after 1-MA treatment, the nuclear envelope breaks down and the nucleoplasm disperses uniformly through the oocyte. This intermixing of nucleoplasm and the cytoplasm renders the oocyte fertilized. BAPTA injected into the nucleus prevents this intermixing. The effect of nuclear BAPTA injection one hour after addition of 1-MA is shown in Fig. 4. In this case the nuclear Ca^{2+} increase was inhibited, and although the first cytoplasmic Ca^{2+} transient (cyto1) was not affected, the dissolution of the nuclear envelope and the reinitiation of meiosis did not occur. In BAPTA-injected nuclei, the nucleoplasm remained in the same location as in the immature oocyte. (Fig. 4A). Electron microscopy of the nuclear envelope in these oocytes showed that it is intact. (Fig. 4B).

We infer from these results that the nuclear Ca^{2+} concentration is regulated depending on the changes of the cytosolic Ca^{2+}, and that Ca^{2+} can diffuse from the cytosol into the nucleus and vice-versa through the nuclear envelope after the addition of 1-MA. It has been shown recently in somatic cells that large (> 600 nM) cytosolic Ca^{2+} increases are attenuated in the nucleus (21). In BALB/c 3T3 cells stimulated to proliferate with platelet derived growth factor (PDGF), the nuclear Ca^{2+} passively followed the changes in cytoplasmic Ca^{2+} induced by PDGF, suggesting that the increase in nuclear Ca^{2+} may contribute to the stimulation of

mitogenesis by PDGF (22,23). Moreover, the presence in the nuclear envelope of a Ca^{2+} ATPase (24), and of IP3-dependent (25) Ca^{2+} channels favors the model that a Ca^{2+} transport system is located in the nuclear envelope.

In summary, we have shown that the increase in baseline nuclear Ca^{2+} is regulated by 1-MA. Furthermore, the baseline increase in nuclear Ca^{2+} is essential for the NEBD. Clamping nuclear Ca^{2+} blocks the continuation of meiosis. Starfish oocytes entering meiosis respond to external signals of such hormones. Thus our results provide support for the concept that a signal transduction system involving Ca^{2+}, related to cell cycle events, is located within the nuclear envelope.

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References

1. Berridge, M. J. (1984) Biochem. J. 220, 345-360.
2. Whitaker, M. J., and Steinhardt, R. A. (1985) In Biology of Fertilization, (C. B. Metz, and A. Monroy, Eds.), vol.III, 167-221. Academic Press, New York.
3. Shen, S. S. (1992) Curr. Op. Gen. Dev. 2, 642-646.
4. Whitaker, M., and Patel, R. (1990) Development 108, 525-542.
5. Poenie, M., Alderton, J., Tsien, R. Y., and Steinhardt, R. A. (1985) Nature 315, 147 149.
6. Twigg, J., Patel, R., and Whitaker, M. (1988) Nature 332, 366-369.
7. Bachs, O., Agell N., and Carafoli, E. (1992) Biochim. Biophys. Acta 1113, 259-270.
8. Dalcq, A. (1925) Arch. Biol. 34, 507-674.
9. Pasteels, J. (1935) Arch. Biol. 46, 229.
10. Kanatani, H., Shirai, H., Nakanishi, K., and Kurosawa, T. (1969) Nature 221, 273-274.
11. Kanatani, H. (1985) In Biology of Fertilization, (C. B. Metz and A. Monroy, Eds.), vol. I, pp. 119-140. Academic Press, New York.
12. Kanatani, H., and Shirai, H. (1967) Nature 216, 284-286.
13. Stevens, M. (1979) Exp. Cell Res. 59, 482-484.
14. Masui Y., and Clarke, H. J. (1979) Int. Rev. Cytol. 57, 185-282.
15. Meijer L., and Guerrier, P. (1984) Int. Rev. Cytol. 86, 129-196.
16. Moreau, M., Guerrier, P., Doree, M., and Ashley, C. C. (1978) Nature 272, 251-253.
17. Picard, A., and Doree, M. (1983) Exp. Cell Res. 145, 325-323.
18. Eisen, A., and Reynolds, G. J. (1984) J. Cell Biol. 99, 1878-1882.
19. Witchel, H. J., and Steinhardt, R. A. (1990) Dev. Biol. 141, 393-398.
20. Hiramoto, H. (1974) Exp. Cell Res. 87, 403-406.
21. Al-Mohanna, F. A., Caddy, K. W. T., and Bolsover, S. R. (1994) Nature, 367, 745-750.
22. Tucher, R. W., and Fay, F. S. (1990) Eur. J. Cell Biol. 51, 120-127.
23. Waybill, M. M., Yelamarty, R. V., Zhang, Y., Scaduto, R. C., LaNou, K. F., Hsu, C., Smith, B. C., Tillotson, D. L., Yu, F. T., and Cheung, J. Y. (1991) Am. J. Physiol. 261, E49-57.
24. Nicotera, P., McConkey, D. J., Jones, D. P., and Orrenius, S. (1989) Proc. Natl. Acad. Sci. USA 86, 453-457.
25. Ross, C. A., Meldolesi, J., Milner, T. A., Satoh, T., Supattapone, S., and Snyder, S. H. (1989) Nature 339, 468-470.