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Activation of Ascidian Eggs

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Changes in $[Ca^{2+}]_i$ are an essential factor regulating egg activation. Matured ascidian eggs are arrested at metaphase I, and two series of $[Ca^{2+}]_i$ transients have been observed after fertilization: Ca^{2+} waves just after fertilization (Series I) and $[Ca^{2+}]_i$ oscillation between the first and second polar body extrusion (Series II). We investigated mechanisms involved in the elevation of $[Ca^{2+}]_i$ and the role of the $[Ca^{2+}]_i$ transients during egg activation in *Ciona savignyi.* The monoclonal antibody 18A10 against IP_3 receptor type 1, which inhibits IP_3-induced Ca^{2+} release in hamster and mouse eggs, did not show substantial inhibitory effects on series I or egg deformation, whereas Series II and the first cell division were inhibited by the antibody. Ruthenium red, an inhibitor of ryanodine receptor-mediated Ca^{2+} release, had no apparent effect of $[Ca^{2+}]_i$ transients and other events related to the egg activation. Microinjection of IP_3 into unfertilized eggs induced $[Ca^{2+}]_i$ transients similar to those seen in Series I, whereas injection of cyclic ADP ribose, an agonist of ryanodine receptors, rarely induced $[Ca^{2+}]_i$ transient. Adenophostin B, a potent nonmetabolizable agonist of IP_3 receptors, induced $[Ca^{2+}]_i$ oscillations which continued after first polar body extrusion, without separation to two series, and led to extrusion of first and second polar bodies. These results suggest that Series II is driven by the mouse type 1-like IP_3 receptor while Series I seems to be mediated by another type of IP_3 receptor. Injection of IP_3 only induced the first polar body extrusion and the egg was arrested at metaphase II even when a higher amount of IP_3 was injected. On the other hand, reinjection of IP_3 after the first polar body extrusion led to emission of the second polar body. Thus, Series I and II of $[Ca^{2+}]_i$ transients are likely to be required for metaphase–anaphase transition in meiosis.

INTRODUCTION

Intracellular Ca^{2+} is as an essential factor regulating various cellular events. In egg activation, transients or oscillatory rises of $[Ca^{2+}]_i$ in eggs are observed in all species investigated thus far, and the $[Ca^{2+}]_i$ transients seem to be critical for initiation of several events related to egg activation and cell cycle control (Jaffe, 1985; Miyazaki *et al.*, 1993; Whitaker and Patel, 1990; Whitaker and Swann, 1993). In sea urchin eggs, $[Ca^{2+}]_i$ transients correlate with the exocytosis of cortical granules (Whitaker and Baker, 1983; Zimmerberg and Whitaker, 1985) and cell cycle in early cleavage (Ciapa *et al.*, 1994; Poenie *et al.*, 1985; Whitaker and Patel, 1990), and $[Ca^{2+}]_i$ seems to trigger nuclear envelope breakdown (Steinhardt and Alderton, 1988; Twigg *et al.*, 1988).

Although $[Ca^{2+}]_i$ transients derive from the extracellular environment in some protostomes (Jaffe, 1985; Stricker, 1996), most $[Ca^{2+}]_i$ transients in deuterostomes derive from intracellular calcium stores and are regulated by two

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mechanisms: IP_3-induced Ca^{2+} release (IICR) mediated by the IP_3 receptor (IP_3R) and Ca^{2+}-induced Ca^{2+} release (CICR) mediated by the ryanodine receptor (RyR) (Berridge 1993a,b; Miyazaki *et al.*, 1993; Whitaker and Swann, 1993). Though there seems to be no RyR in hamster and *Xenopus* eggs, it does exist in most animal eggs, even in some mammals (Miyazaki *et al.*, 1992; Parys *et al.*, 1992; Whitaker and Swann, 1993; Yue *et al.*, 1995).

Increases of $[Ca^{2+}]_i$ at fertilization are due to both the IP_3R-mediated Ca^{2+} release and the RyR-mediated Ca^{2+} release in sea urchin eggs (Galione *et al.*, 1993; Lee *et al.*, 1993), and the phosphoinositide messenger system oscillates during the early embryonic cell cycle (Ciapa *et al.*, 1994). In the amphibian *Xenopus laevis*, preinjection of heparin, a competitive inhibitor of the IP_3R (Ghosh *et al.*, 1988), arrests $[Ca^{2+}]_i$ transients (Nuccitelli *et al.*, 1993), and injection of IP_3 induces cortical contraction (Kume *et al.*, 1993). Sperm-induced $[Ca^{2+}]_i$ oscillations of hamster eggs are blocked by preinjection of the monoclonal antibody (mAb) 18A10, which recognizes the mouse and the hamster type 1 IP_3R and inhibits the receptor-mediated Ca^{2+} release but not IP_3 binding (Miyazaki *et al.*, 1992; Nakade *et al.*, 1991). 18A10 also inhibits some early and late events of activation of mouse eggs (Xu *et al.*, 1994). Therefore IP_3R-mediated Ca^{2+} release seems to be the main mechanism of $[Ca^{2+}]_i$ elevation in these animals.

Increases in $[Ca^{2+}]_i$ in eggs at fertilization have been observed in some ascidians (Brownlee and Dale, 1990; Russo *et al.*, 1996; Speksnijder *et al.*, 1989, 1990a,b). Unfertilized ascidian eggs are arrested at metaphase I (Dale, 1983), and fertilization induces cytoplasmic movements with egg deformation, ooplasmic segregation, and extrusion of first and second polar bodies (Conklin, 1905; Sardet *et al.*, 1989; Sawada and Osanai, 1981). Egg deformation can be induced by application of the calcium ionophore A23187 or IP_3, without insemination (Bevan *et al.* 1977; Dale, 1988; Jeffery, 1982; Roegiers *et al.*, 1995). Polar body extrusion is prevented by preinjection of low Ca^{2+} buffer and is induced by high Ca^{2+} buffer or ionomysin (McDougall and Sardet, 1995; Sensui and Morisawa, 1996). Russo *et al.* (1996) observed that heparin inhibited $[Ca^{2+}]_i$ oscillation between first and second polar body extrusions (Series II) in the *Ciona* egg. While these events show that Ca^{2+} plays an important role in egg activation, the mechanism of $[Ca^{2+}]_i$ transients and the role of Ca^{2+} in egg activation are unclear.

We investigated mechanisms related to Ca^{2+} mobilization and the role of Ca^{2+} in egg activation, and obtained evidence indicating that $[Ca^{2+}]_i$ transients between extrusion of first and second polar bodies (Series II) are driven by type 1-like IP_3R, while those occurring prior to the first polar body extrusion (Series I) seemed to be mediated by a different mechanism in the ascidian egg. These $[Ca^{2+}]_i$ transients are likely to be required for metaphase–anaphase transition in meiosis.

MATERIALS AND METHODS

Materials

Specimens of the ascidian, *Ciona savignyi*, were collected in Aburatsubo Bay in Kanagawa Prefecture, and in Otsuchi Bay in Iwate Prefecture, Japan. Specimens of sea urchin, *Strongylocentrotus nudus*, collected in Mutsu Bay in Aomori Prefecture, Japan, were kept in aquaria continuously flowing seawater, and *C. savignyi* were stored under constant light to prevent spontaneous spawning. Eggs and sperm of *C. savignyi* were obtained by dissecting the gonoducts. *S. nudus* gametes were obtained by intracoelomic injection of 0.5 M KCl. If necessary, the vitelline coat and follicle cells of *Ciona* egg were removed by manipulation with stainless-steel needles or immersing them in seawater containing 1% sodium thioglycolate and 0.05% actinase E (Kaken Pharmaceutical Co., Tokyo, Japan) (Mita-Miyazawa and Satoh, 1986).

The artificial seawater consisted of 462 mM NaCl, 9 mM KCl, 11 mM $CaCl_2$, 48 mM $MgCl_2$, and 10 mM Hepes–NaOH (pH 8.2). Adenophostin B was a generous gift from Sankyo Co., Ltd. Inositol 1,4,5-trisphosphate (IP_3) was purchased from Dojin Laboratory (Kumamoto, Japan); heparin (low molecular weight: M_r 3000) was from Sigma (St. Louis, MO); ruthenium red was from Research Biochemicals Inc. (Natick, MA); cyclic ADP ribose (cADPR) was from Calbiochem (San Diego, CA); purified rat IgG was from Chemicon International Inc. (Temecula, CA). All chemicals were of analytical grade.

Microinjection

The method of microinjection was much the same as described previously (Deguchi and Osanai, 1994), using a water pressure injector (IM-5B; Narishige, Tokyo, Japan). The volume of the injected solution was estimated by measuring the diameter of droplets injected into mineral oil. Usually the reagents were injected 1/60–1/30 volume of the egg (35–70 pl), except for injections of antibodies. Rat IgG or mAb 18A10 was injected 1/20–1/13 volume of the egg (105–165 pl).

Under Results, the concentration of injecting reagents is usually given as that of injectants.

$[Ca^{2+}]_i$ Measurements with Calcium Green 1 Dextran

Calcium green 1 dextran (M_r 10,000) (CaG1dx; Molecular Probes Inc., Eugene, OR) was dissolved at 250 μM in injection buffer (IB; 50 mM KCl, 100 μM EGTA, 10 mM Hepes–KOH, pH 7.1) and microinjected into eggs to give a cytoplasmic concentration of 5–20 μM. Ruthenium red, mAb 18A10, and rat IgG were coinjected with CaG1dx when the reagents were injected. When other reagents (IP_3, ryanodine, cADPR, adenophostin B) were injected, they were dissolved in an appropriate concentration in IB and injected as described above.

Eggs were exposed to the excitation beam through a 470- to 490-nm band-pass filter for 200 ms at 5-s intervals, and fluorescence images were collected with a 515- to 550-nm band-pass filter on an inverted microscope (IX-70; Olympus, Tokyo, Japan). The images were then transmitted via a digital camera (PXL-37; Photometrics, Tucson, AZ) to a computer (Power Macintosh 9500/120; Apple, Tokyo, Japan) and analyzed with TI Workbench; the original software prepared by T.I. Experiments were conducted at room temperature (ca. 25°C).

Imaging of $[Ca^{2+}]_i$ with Fura 2

Fura 2 (Dojin Laboratory) was dissolved at 1 mM in IB and injected into the naked egg as described above (cytoplasmic concentration: 16–34 μM). The eggs were excited at 340 and 380 nm for 200 ms each at 5-s intervals, and fluorescence images after the 510-nm high-pass filter were collected as described above. Ratio of the fluorescence intensities at 340 nm (F340) and at 380 nm (F380) was calculated as relative $[Ca^{2+}]_i$ concentrations.

Microtubule and DNA Staining

Naked eggs were obtained as described above. Eggs were fixed for 1–2 h in solution containing 4% paraformaldehyde (Wako Pure Chemicals Inc., Osaka, Japan), 0.25% glutaraldehyde (Nacalai Tesque, Kyoto, Japan), 80 mM Pipes–KOH, pH 6.8, 1 mM $MgCl_2$, 5 mM EGTA, and 0.2% Triton X-100 at room temperature, and then incubated overnight in absolute methanol at −30°C. After fixation, eggs were washed twice for 15 min each in Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris–HCl, pH 7.4) containing 0.1% Triton X-100 (T-TBS). The primary antibody was 0.5 μg/ml anti-α tubulin antibody (Cappel, Durham, NC) diluted in T-TBS. Following incubation with the primary antibody (1.5 h), eggs were washed three times for 10 min each with T-TBS, incubated for 1 h in the secondary antibody (FITC-conjugated goat anti-mouse IgG; Mo Bio Lab. Inc., Solana Beach, CA) diluted 1:200 in T-TBS with 0.5 μg/ml DAPI (Molecular Probes), and then washed three times for 10 min each in TBS. Eggs were examined under a fluorescence microscope (IX-70; Olympus).

Immunolocalization of the IP_3 Receptor in Eggs

Naked eggs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (200 mM NaCl in 13 mM phosphate buffer, pH 7.4) for 1–2 h; then the eggs were washed three times for 10 min each with PBS containing 0.1% polyvinylalcohol and incubated in blocking buffer (PBS containing 3% BSA) for 1 h. The eggs were next incubated for 2 h in 100 μg/ml of mAb 18A10 or in 100 μg/ml of rat IgG diluted in blocking buffer and then washed in blocking buffer three times for 10 min each. The eggs were incubated for 1 h in the second antibody (FITC-conjugated goat anti-rat IgG, Cappel Products) diluted 1:200 in blocking buffer and then washed three times for 10 min each in PBS. The eggs were examined using a laser-scanning confocal microscope (Fluoview; Olympus).

Preparation of Crude Membrane Proteins and Western Blotting

Crude membrane proteins were preparated as described previously (Kume *et al.*, 1993). Crude membrane proteins were electrophoresed in 5% SDS–polyacrylamide gel by the method of Laemmli (1970) and transferred to a hydrophobic polyvinylidine difluoride membrane. The blots were stained with Coomassie brilliant blue R-250 or with 10 μg/ml mAb 18A10 and 10 μg/ml rat IgG for 1.5 h at room temperature, followed by incubation with 1:500 diluted horseradish peroxidase-conjugated anti-rat immunoglobulin (Amersham, Buckinghamshire, England) as secondary antibody, and visualized with ECL Western blotting detection reagents (Amersham).

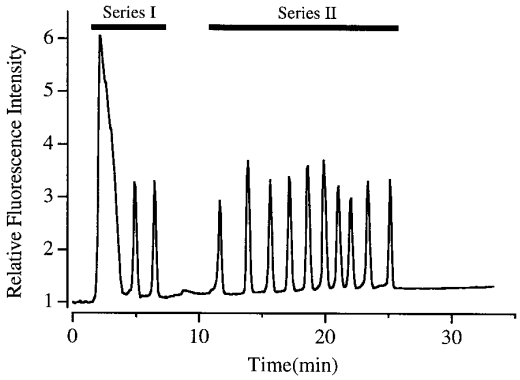


FIG. 1. Typical $[Ca^{2+}]_i$ changes in an inseminated egg of *Ciona savignyi*. Intracellular Ca^{2+} changes were analyzed using fura 2. There were two series of $[Ca^{2+}]_i$ transients. The first series of $[Ca^{2+}]_i$ transients (shown as Series I) consisted of one large initial wave and 1–4 small waves, and lasted 5.1 ± 0.1 min, while second series of $[Ca^{2+}]_i$ transients (shown as Series II) was observed 4.5 ± 0.2 min after the end of Series I and continued for 11.1 ± 0.3 min and consisted of 8–14 transients.

Data Presentation

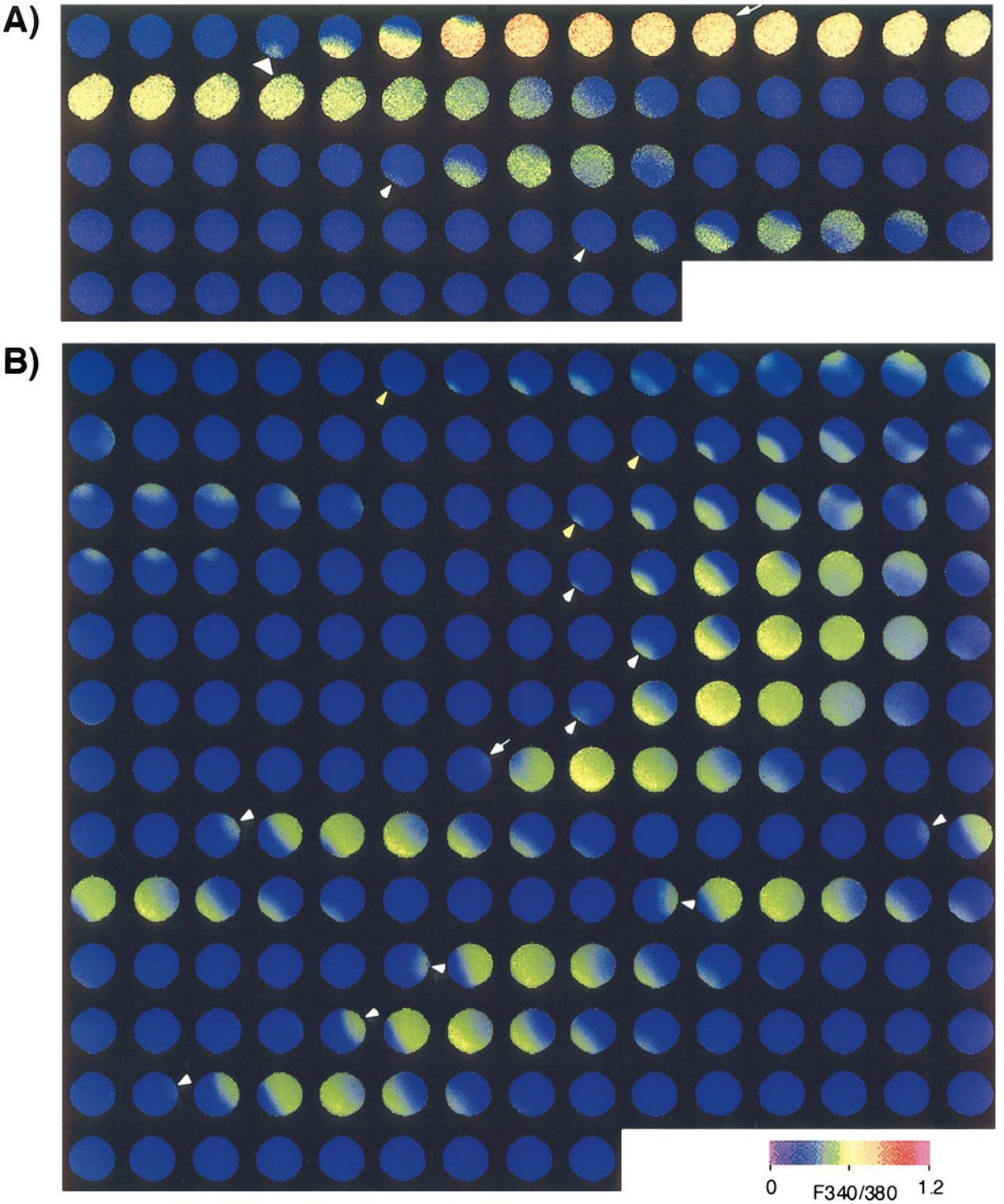
Under results, data represent averages of several experiments and are usually plotted as means ± SEM.

RESULTS

Calcium Waves and Calcium Oscillation in the Inseminated Egg

The first, the spatiotemporal patterns of $[Ca^{2+}]_i$ in the ascidian egg were analyzed using our calcium imaging system with fura 2 and CaG1dx. CaG1dx is a useful Ca^{2+} indicator because it is excited by visible wavelength lights and has less toxicity. However, it is not useful for examining distribution of Ca^{2+} in cells because the use of ratiomatic measurements is impossible in case of the indicator alone. On the other hand, fura 2 is a ratiometric Ca^{2+}

FIG. 2. Pseudo-colored ratioed images showing Ca^{2+} dynamics of an inseminated egg of *Ciona savignyi*. The experiments were performed on the same egg as for Fig. 1. Blue indicates relatively low F340/F380 ratios of fura 2 and $[Ca^{2+}]_i$ concentration, whereas yellow and red indicate higher F340/F380 ratios and Ca^{2+}. Images were collected every 5 s and are ordered left-to-right and top-to-bottom. (A) Ca^{2+} dynamics of Series I. The Ca^{2+} wave was propagated from an estimated sperm entry point soon after insemination (thick arrowhead), and



small Ca^{2+} waves which were propagated from the thin arrowhead followed. Egg deformation was observed 33 ± 1 s after the first Ca^{2+} wave (arrow). (B) Ca^{2+} dynamics of Series II. Transients of $[Ca^{2+}]_i$ look like waves propagated from an arrowhead. Direction of the Ca^{2+} waves of Series II was reversed once or twice (arrow). Ca^{2+} waves of early transients in Series II (early 3 transients in this experiment) were weak and sometimes propagated incompletely (yellow arrowhead).

indicator, but it is excited by UV lights and has toxicity. Therefore, we used CaG1dx to measure $[Ca^{2+}]_i$ of the whole egg and fura 2 for examination Ca^{2+} distribution in the egg. The pattern of $[Ca^{2+}]_i$, measured based on these two indicators showed a similar tendency. As shown in Fig. 1, two series of $[Ca^{2+}]_i$ transients were observed in the inseminated egg, much the same as seen with *C. intestinalis* (Russo *et al.*, 1996). The first series of $[Ca^{2+}]_i$ transients (Series I) consisted of one large initial transient which occurred soon after insemination and following 1–4 (1.8 ± 0.2, $n = 19$) small transients. Series I lasted for 5.1 ± 0.1 min (Fig. 1), and all $[Ca^{2+}]_i$ transients were in the form of waves (Fig. 2A). All Ca^{2+} waves of Series I were usually propagated from the same point, assumed to be the sperm entry point. The wave velocity of the initial transient is 6.2 ± 0.3 μm/s ($n = 17$), and that of small transients is 10.2 ± 0.9 μm/s ($n = 8$). Egg deformation was observed 33 ± 1 s ($n = 16$) after the start point of Series I (Fig. 2A, arrow). The second series of $[Ca^{2+}]_i$ transients (Series II) was observed 4.5 ± 0.2 min after the end of Series I (Fig. 1), lasted 11.1 ± 0.3 min, and consisted of 8–14 transients (mean 11.7 ± 0.4, $n = 19$). These transients also appeared as waves (Fig. 2B). Usually direction of the Ca^{2+} waves of Series II was reversed once or twice (arrow in Fig. 2B). Ca^{2+} waves of early transients in Series II were weak and sometimes propagated incompletely (yellow arrowheads in Fig. 2B).

Role of IP_3R-Mediated Ca^{2+} Release on the Egg Activation

The mAb 18A10 (Maeda *et al.*, 1988) binds to an epitope of 12 amino acid residues at the carboxy terminus of the mouse IP_3R type 1, which is included in the proposed Ca^{2+} channel region, and block IICR but not IP_3 binding (Furuichi *et al.*, 1989; Nakade *et al.*, 1991). First, we asked if the mAb 18A10 would cross-react with the IP_3R in the egg of *C. savignyi*. The mAb 18A10 stained the cortical area and inner cytoplasm, and 260- and 95-kDa proteins (Fig. 3), suggesting that the IP_3R to which mAb 18A10 binds is expressed in the egg. Effects of mAb 18A10 on egg activation were next investigated. When mAb 18A10 was injected into the eggs prior to insemination, Series I of $[Ca^{2+}]_i$ transients and the egg deformation did not seem to be inhibited, but Series II was abolished (10/14) (Fig. 4A), or number of the $[Ca^{2+}]_i$ transients in Series II was reduced (3/14) (Fig. 4B). In both cases, second polar body formation and first cleavage were not observed. Injection of rat IgG had no apparent effect on $[Ca^{2+}]_i$ transients (Series I and II) and egg activation (Fig. 4C). In other ascidians, Series II of $[Ca^{2+}]_i$ transients was also suppressed by heparin which is antagonist of all known IP_3R (McDougall and Sardet, 1995; Russo *et al.*, 1996). In our experiments, the egg of *C. savignyi* into which 23 mg/ml heparin had been injected showed Series I, but egg deformation and Series II were not observed (data not shown). These results suggest that IP_3R exists in the ascidian egg and the IP_3R-mediated Ca^{2+} release participates in the control of egg activation.

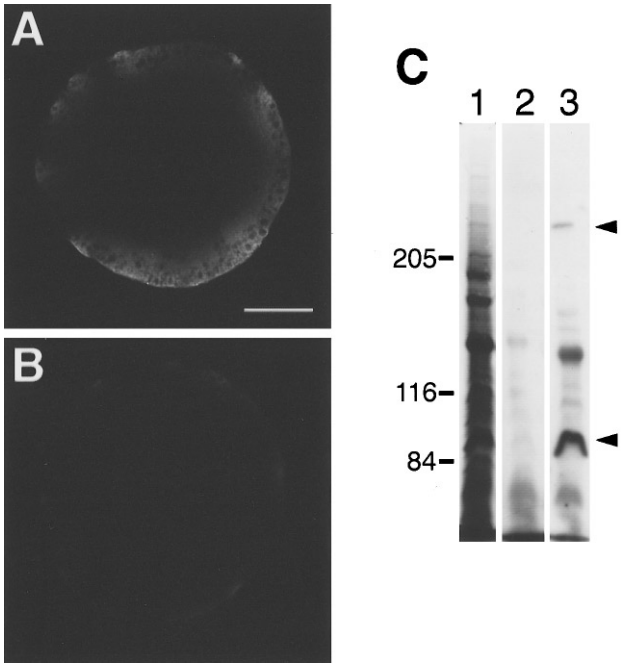


FIG. 3. Cross-reactivity of mAb 18A10 on the egg of *Ciona savignyi*. Immunostaining of whole *Ciona* eggs with (A) the mAb 18A10 or (B) rat IgG as a negative control. Eggs were examined using a laser-scanning confocal microscope. mAb 18A10 stained the cortical area and inner cytoplasm. Bar represents 100 μm. (C) Western blots of crude membrane proteins from *Ciona* eggs (14 μg) using mAb 18A10 and purified rat IgG. Proteins were separated by 5% SDS–PAGE, and stained with Coomassie Brilliant Blue R-250 (lanes 1) or detected on immunoblot analysis with rat IgG (lanes 2) and mAb 18A10 (lanes 3). Arrowheads indicate the proteins of *Ciona* eggs on which 18A10 cross-reacted.

Next, we wanted to observe if IP_3 would induce $[Ca^{2+}]_i$ transients and events related to egg activation. Injection of 5–100 μM IP_3 into unfertilized eggs led to a Series I-like $[Ca^{2+}]_i$ transients; one large initial $[Ca^{2+}]_i$ transient and 1–2 transients (5 μM, $n = 6$; 20 μM, $n = 14$; 100 μM, $n = 3$) (Fig. 5A). In such cases, Series II-like $[Ca^{2+}]_i$ transients were not observed (Fig. 5A). Injection of the injection buffer into the egg has no apparent effects on $[Ca^{2+}]_i$ transients and egg activation (data not shown). Induction of Series I-like $[Ca^{2+}]_i$ transients by injection of 5 μM IP_3 was not affected by preinjection of heparin up to 23 mg/ml, although egg deformation was prevented (6/6) (Fig. 5B). IP_3-induced $[Ca^{2+}]_i$ transients was not prevented by 18A10 up to 1.8 mg/ml, the upper limit of available concentration, either (2/2) (Fig. 5C). The presence of a heparin-insensitive IP_3 receptor in the ascidian egg would need to be considered.

Role of RyR-Mediated Ca^{2+} Release on the Egg Activation

Effects of RyR-mediated Ca^{2+} release on activation of ascidian eggs were next investigated. Ruthenium red is an

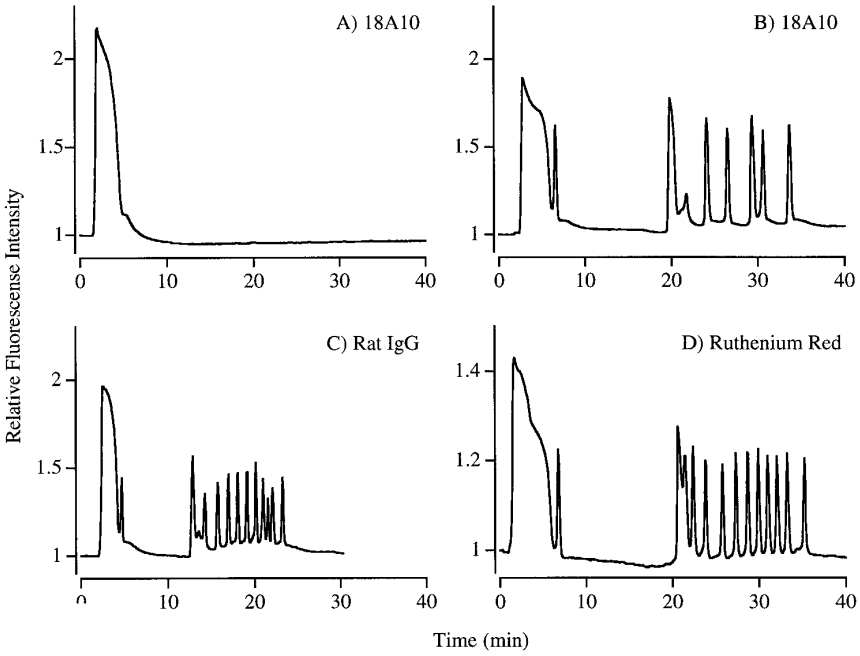


FIG. 4. Inhibition of $[Ca^{2+}]_i$ changes in the fertilized eggs of *Ciona savignyi* by injection of (A, B) 1.8 mg/ml 18A10, (C) 2.4 mg/ml control rat IgG, or (D) 2.3 mg/ml ruthenium red with CaG1dx prior to insemination. Series II was abolished (A) (10/14) or number of the $[Ca^{2+}]_i$ transients in Series II was reduced (B) (3/14) by mAb 18A10. The estimated intracellular concentrations of mAb 18A10 (A, B), rat IgG (C), and ruthenium red (D) are 90–140, 120–180, and 38–77 μg/ml, respectively.

inhibitor of RyR and blocks RyR-mediated Ca^{2+} release (Fleischer and Inui, 1989). Microinjection of ruthenium red had no apparent effect on $[Ca^{2+}]_i$ transients (Fig. 4D), egg deformation, polar body extrusion, and cleavage (9/10), but a delay in the schedule occurred, as follows: On ruthenium red-injected eggs, Series II of $[Ca^{2+}]_i$ transients began 15.6 $\pm$ 0.7 min after fertilization and lasted 14.0 $\pm$ 0.5 min (n = 9), whereas it began 9.5 $\pm$ 0.2 min after fertilization and lasted 11.1 $\pm$ 0.3 min in case of a normal development of the egg. First cleavage was usually observed at 45–50 min after fertilization, while it was observed at 70–80 min in ruthenium red-injected eggs.

Cyclic ADP ribose (cADPR) is an agonist of RyR and it induces RyR-mediated Ca^{2+} release (Berridge, 1993b; Galione *et al.*, 1991). First, cADPR was injected into the eggs of the sea urchin, *S. nudus,* an animal known to have the RyR and be sensitive to cADPR (Galine *et al.*, 1993; Lee *et al.*, 1993), for the positive control. Injection of 500 μM cADPR into the sea urchin eggs induced $[Ca^{2+}]_i$ transients and elevation of the fertilization envelope (7/7), and 50 μM cADPR also elevated $[Ca^{2+}]_i$ and the fertilization envelope (n = 2). On the other hand, when cADPR was injected into the ascidian eggs, $[Ca^{2+}]_i$ transient was usually not observed (12/17) (Fig. 5D), and even when a $[Ca^{2+}]_i$ transient did occur, small transients following the initial $[Ca^{2+}]_i$ transient were not observed (5/17) (Fig. 5E). These results suggest that RyR-mediated Ca^{2+} release participants little in egg activation and that IP_3R-mediated Ca^{2+} release is probably the main mechanism of $[Ca^{2+}]_i$ elevation in the ascidian egg.

IP_3-Induced Ca^{2+} Transients Drive Meiosis

Injection of IP_3 also led to morphological changes. The egg in which 50 μM IP_3 was injected was deformed and extruded the first polar body at 10–11 min after the injection of IP_3, but extrusion of the second polar body was not observed for at least 60 min after the injection (8/8) (Table 1). Second polar body formation was induced only by reinjection of IP_3 into IP_3-injected eggs at 17–27 min after first injection (8/10) (Table 1). In this case, reinjection of IP_3 did not led to Series II-like $[Ca^{2+}]_i$ transients, but only Series I-like $[Ca^{2+}]_i$ transients were seen (Fig. 5F). Injection of a lower dose of IP_3 (20 μM) in two steps also induced extrusion of first and second polar bodies (Figs. 6Ab, and 6Ac), while a single injection of a higher dose of IP_3 (100 μM) into unfertilized eggs induced only extrusion of first polar body (7/7) (Table 1). These results suggest that extrusion of first and second polar bodies is controlled by two series of $[Ca^{2+}]_i$ transients, which may be caused by IP_3R-mediated Ca^{2+} release. Elevation of $[Ca^{2+}]_i$ after the first polar body extrusion may be necessary for extrusion of the second polar body.

The stage of meiosis in the egg after IP_3 injection was

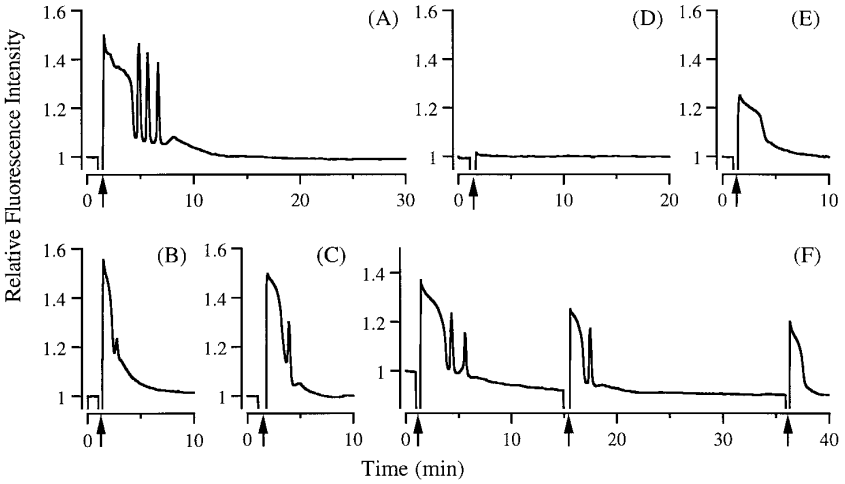


FIG. 5. Intracellular Ca^{2+} release in unfertilized eggs, based on fluorescence changes in CaG1dx. The following compounds were injected into the eggs at the time indicated by arrows: (A, F) 20 μM IP_3, (B, C) 5 μM IP_3, (D, E) 500 μM cADPR. The estimated final concentrations of reagents are 330–670 nM (A, F), 90–170 nM (B, C), and 9–17 μM (D, E). Injection of IP_3 induced Series I-like $[Ca^{2+}]_i$ transients (A). (B, C) Prior to IP_3 injection, 23 mg/ml heparin (final 380–770 μg/ml) (B) or 1.8 mg/ml mAb 18A10 (final 90–140 μg/ml) (C) had been injected into the eggs. Heparin and 18A10 had no apparent effects on IP_3-induced $[Ca^{2+}]_i$ transients. When cADPR was injected into the eggs, usually $[Ca^{2+}]_i$ transients were not observed (D) (12/17), and small transients were not observed even if cADPR injection led to a $[Ca^{2+}]_i$ transient (E) (5/17). (F) Intracellular Ca^{2+} changes by repetitive injection of IP_3. Second or third injections of IP_3 did not induce Series II-like $[Ca^{2+}]_i$ transients, but only Series I-like $[Ca^{2+}]_i$ transients.

next examined. Extrusion of the first polar body was usually observed 7–9 min after the IP_3 injection. At 10 min after the injection, spindle-like structures were not observed, but chromosomes and polar microtubules were observed, indicative of telophase I (15/16) (Figs. 7A and 7B). At 15 min after the injection, chromosomes and spindle-like structures were observed (9/14) (Figs. 7C and 7D). Chromosomes still seemed to be aligned along the equatorial plane 60 min after the injection, although alignment of the chromosomes and spindles in some eggs partially collapsed (3/7) (Figs. 7E and 7F). The results of these experiments are summarized in Fig. 8. Our observations show that the nucleus state of IP_3-injected egg becomes metaphase II within 15 min and that meiosis stops again at metaphase II. Next we observed at what point Ca^{2+} was required in the second polar body extrusion. When intervals between two IP_3 injections were changed in steps, extrusion of the second polar body was observed only when the second injection was given 15–30 min after the first one (Figs. 6A and 6B). Therefore it seems that $[Ca^{2+}]_i$ transients drive the metaphase–anaphase transition in meiosis.

TABLE 1

Rate of Extrusion of First and Second Polar Bodies Induced by IP_3 Injection into Eggs

1st injection	2nd injection	1st polar body	2nd polar body
800 nM IP_3[a]	None	100% (8/8)	0% (0/8)
800 nM IP_3	800 nM IP_3	100% (10/10)	80% (8/10)
800 nM IP_3	Injection buffer	100% (7/7)	0% (0/7)
1600 nM IP_3	None	100% (7/7)	0% (0/7)

[a] Concentration of reagents is given as the final concentration in the eggs. See Materials and Methods.

Effects of Adenophostin B

Adenophostin B is a nonmetabolizable agonist of the IP_3R, and has higher binding affinity and Ca^{2+} release activity than the native ligand, IP_3 (Hirota *et al.*, 1995; Takahashi *et al.*, 1993, 1994). Because it can induce $[Ca^{2+}]_i$ oscillations in the mouse egg (Sato *et al.*, 1998), it is a significant tool to investigate the role of IP_3R-mediated Ca^{2+} release. Injection of adenophostin B into the *Ciona* egg induced $[Ca^{2+}]_i$ oscillations which continued for at least 30 min (Fig. 9). Oscillations of $[Ca^{2+}]_i$ also propagated as waves (data not shown). The interval between $[Ca^{2+}]_i$ transients in the oscillation induced by adenophostin was 164 ± 14 s (n = 11), while the interval between $[Ca^{2+}]_i$ transients in the oscillation in Series II was 58 ± 10 s (n = 19), and the Ca^{2+} wave induced by adenophostin seemed to propagate faster than in case of normal insemination. It is possible that higher binding affinity to IP_3R leads to fast propagation of Ca^{2+} wave and long-term $[Ca^{2+}]_i$ uptake.

In addition, adenophostin also induced egg deformation and extrusion of first and second polar bodies without

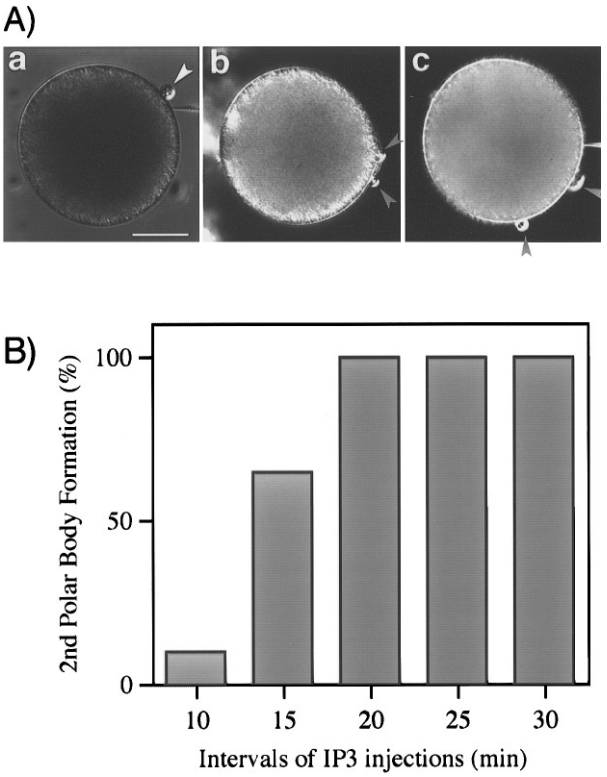


FIG. 6. Induction of second polar body extrusion by two injections of 20 μM IP_3 (final 330–670 nM). (A) The second injection of IP_3 was given at (a) 10 min, (b) 15, in, or (c) 20 min after the first injection. In all cases, first polar body extruded before the second injection. Injection of IP_3 induced first polar body extrusion. Second polar body was extruded in b and c (arrowhead). Bar represents 100 μm. (B) Relation between the intervals of IP_3 injection and second polar body extrusion. The second polar body extrusion was induced when the second injection was given over 15 min after the first injection.

cessation of the oscillation of $[Ca^{2+}]_i$. An egg injected with 1 μM adenophostin B extruded two polar bodies within 1 h, while 100 μM IP_3 induced only one polar body extrusion. These results support the notion that $[Ca^{2+}]_i$ transients after the first polar body extrusion are required for second body extrusion.

DISCUSSION

Roles of IP_3R-Mediated Ca^{2+} Release and RyR-Mediated Ca^{2+} Release in Egg Activation

Mobilization of Ca^{2+} from internal stores is regulated by IP_3R and/or RyR (Berridge, 1993a,b; Furuichi *et al.*, 1994; Furuichi and Mikoshiba, 1995; Miyazaki *et al.*, 1993; Whitaker and Swann, 1993). In sea urchin eggs, $[Ca^{2+}]_i$ increases at fertilization involve both mechanisms (Galione *et al.*, 1993; Lee *et al.*, 1993). However, the existence of the RyR has not been observed in hamster and *Xenopus* eggs (Miyazaki *et al.*, 1992; Nuccitelli *et al.*, 1993; Parys *et al.*, 1992; Whitaker and Swann, 1993), and there is no evidence that RyR-mediated Ca^{2+} release is involved in egg activation in other animals. In the ascidian egg, the existence of the RyR-mediated Ca^{2+} release was noted in *C. intestinalis* (Arnoult *et al.*, 1997). Another group of workers reported that ryanodine or cADPR, which are agonists of RyR, did not trigger $[Ca^{2+}]_i$ transients in the ascidian *Phallusia mammillata* (Albrieux *et al.*, 1997; McDougall and Sardet, 1995). We found that cADPR induced $[Ca^{2+}]_i$ transients, but the mobilization of Ca^{2+} and egg deformation induced by cADPR was low even when a high concentration of cADPR was injected. In addition, ruthenium red had no apparent effect on $[Ca^{2+}]_i$ transients, while mAb 18A10 inhibited $[Ca^{2+}]_i$ transients in Series II. It is probable that IP_3R-mediated Ca^{2+} release is the main mechanism of $[Ca^{2+}]_i$ elevation in activation of the *Ciona* egg and that participation of RyR-mediated Ca^{2+} release is little.

Mechanisms of Series I and II of $[Ca^{2+}]_i$ Transients

Series I, especially the initial $[Ca^{2+}]_i$ transient, was not inhibited by mAb 18A10, by heparin or ruthenium red, but Series II was suppressed by mAb 18A10 and by heparin. Therefore, Series I may not be driven by IP_3R- or RyR-mediated Ca^{2+} release, while Series II is mediated by IP_3R-mediated Ca^{2+} release. In the nemertean worm and the bivalve, *Mytilus edulis*, extracellular Ca^{2+} entry causes

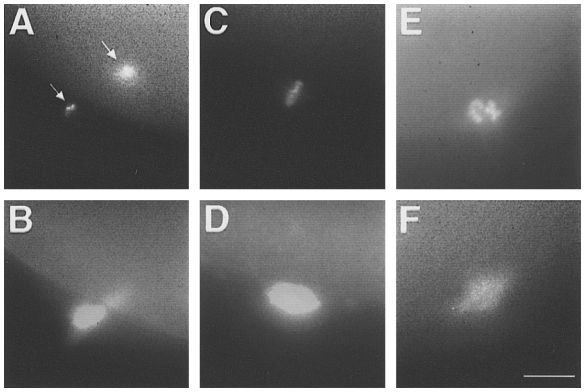
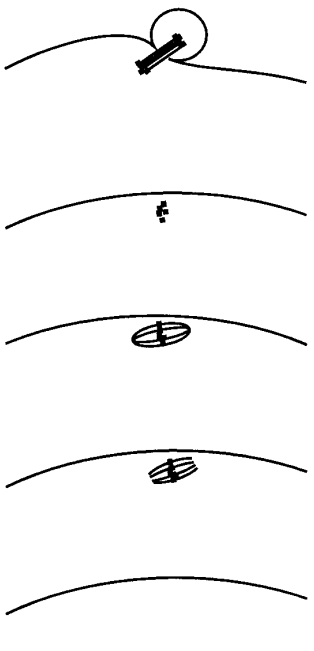


FIG. 7. State of the nucleus in the eggs at (A, B) 10 min, (C, D) 15 min, or (E, F) 60 min after the IP_3 injection. Eggs into which 50 μM IP_3 (final 0.9–1.7 μM) had been injected were fixed at each time and stained with (A, C, E) DAPI or (B, D, F) anti-tubulin antibody. At 10 min after injection, two sets of chromosomes were seen even when one was out of focus (A, arrow), and polar microtubules were observed (B). At 15 and 60 min after the injection, spindle-like structures were observed (D, F) and chromosomes seemed to be aligned along the equatorial plane (C, E). The alignment of the chromosomes partially collapsed 60 min after injection (E). Bar indicates 10 μm.



	Time after IP_3 injection		
	10min (n=16)	15min (n=14)	60min (n=7)
Telophase I (Chromosomes and polar microtubules were observed.)	94%	0%	0%
Prophase II? (Chromosomes, but not microtubules were observed.)	0%	21%	0%
Metaphase II (Chromosomes and spindle-like structures were observed.)	0%	64%	0%
Metaphase II? (Chromosomes and spindle-like structures were observed, although alignment of the chromosomes and spindles partially collapsed in some eggs.)	0%	0%	43%
Unknown (Chromosomes and microtubules were not observed.)	6%	14%	57%

FIG. 8. Schematic drawing of the stage of meiosis and distribution of the stage in the IP_3-injected egg at 10, 15, and 60 min after the injection. Mature oocytes of the ascidian egg usually arrested in metaphase I. At 10 min after the injection of IP_3, a telophase I-like structure was observed in almost all eggs (15/16), and a metaphase II-like structure was observed at 15 min after the injection (9/14). A metaphase II-like structure was seen 60 min after the injection, although alignment of chromosomes and spindles partially collapsed (3/7).

initial $[Ca^{2+}]_i$ transients at fertilization, although in these cases, the $[Ca^{2+}]_i$ transient is not seen as a wave but as a cortical flash (Deguchi *et al.*, 1996; Stricker, 1996). Another notion is that both IP_3R and RyR act on the Ca^{2+} wave. Actually, the $[Ca^{2+}]_i$ transient is controlled by both receptors and cannot be inhibited in the sea urchins even when one receptor was blocked (Galione *et al.*, 1993; Lee *et al.*, 1993). In the ascidian, RyR-mediated Ca^{2+} release may not be the main mechanism in formation of the Ca^{2+} wave because injection of cADPR little elevated $[Ca^{2+}]_i$ in the egg and yet caused activation. Furthermore, we found that injection of IP_3 mimicked Series I, and that heparin and 18A10 did not have inhibitory effects on IP_3-induced $[Ca^{2+}]_i$ transients. Thus, it is likely that there is another type of IP_3R which is insensitive to 18A10 or heparin and which causes Series I. Soluble sperm proteins which can trigger $[Ca^{2+}]_i$ transients in eggs have been found in the hamster and in the nermertean worm (Parrington *et al.*, 1996; Stricker, 1997), so it is possible that Series I is driven by a pathway mediated by the sperm factor.

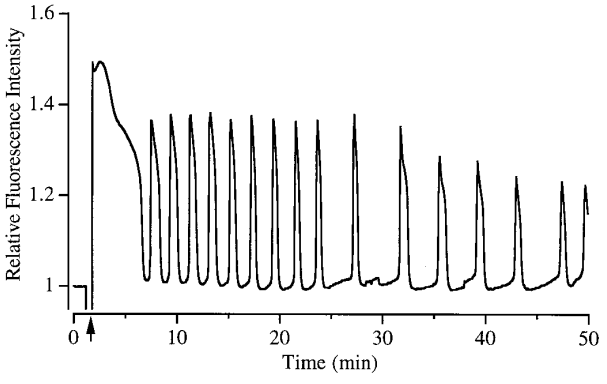


FIG. 9. Intracellular Ca^{2+} changes by injection of adenophostin B into the *Ciona* egg. 1 μM adenophostin B was injected at the time indicated by the arrow. Oscillation of $[Ca^{2+}]_i$ continued for at least 30 min. The estimated cytoplasmic concentration of adenophostin B is 17–33 nM.

Effects of mAb 18A10 and Differences from That of Heparin

18A10 is a monoclonal antibody against the mouse type 1 IP_3R and inhibits Ca^{2+} release from the internal store in mouse and hamster eggs (Mehlmann *et al.*, 1996; Miyazaki *et al.*, 1992; Xu *et al.*, 1994). In the present work, mAb

18A10 inhibited Series II of $[Ca^{2+}]_i$ transients, but it had no apparent effect on Series I and egg deformation in the ascidian egg. On the other hand, heparin blocked small $[Ca^{2+}]_i$ transients in Series I and egg deformation (Russo *et al.*, 1996). This discrepancy can be explained by the finding that mAb 18A10 inhibits only the IP_3R type 1 while heparin is thought to inhibit many subtypes of the IP_3R. Although subtypes of IP_3R in the ascidian are still unknown, it is possible that egg deformation is controlled by other subtypes of IP_3R rather than the one which is sensitive to 18A10. In addition, it is likely that the inhibition of egg deformation by heparin is not due to IICR inhibition but rather to side effects of heparin because heparin has side effects, e.g., inhibition of protein kinase C (Herbert *et al.*, 1996; Herbert and Maffrand, 1991; Wright *et al.*, 1989).

Role of $[Ca^{2+}]_i$ Transients in the Cell Cycle

The relation between Ca^{2+} and the cell cycle (meiosis or mitosis) has attracted much attention (Means, 1994; Whitaker and Patel, 1990). Transients of $[Ca^{2+}]_i$ and the phosphoinositide messenger system correlate to the cell cycle at early cleavage (Ciapa *et al.*, 1994; Poenie *et al.*, 1985; Whitaker and Patel, 1990), and Ca^{2+} seems to trigger nuclear envelope breakdown (Steinhardt and Alderton, 1988; Twigg *et al.*, 1988), in the sea urchin eggs. In zebrafish and *Xenopus* eggs, $[Ca^{2+}]_i$ transients were observed at the cleavage furrow in the early embryonic cell cycle (Chang and Meng, 1995; Muto *et al.*, 1996). In the ascidian egg $[Ca^{2+}]_i$ transients probably control meiosis. We found that injection of IP_3 led to progression of the meiotic cell cycle from metaphase I to metaphase II, and that reinjection of IP_3 at metaphase II stage after extrusion of the first polar body is necessary for completion of meiosis in the ascidian *C. savignyi*. Extrusion of the polar body is prevented by preinjection of low Ca^{2+} buffer, and it is induced by injection of high Ca^{2+} buffer, without fertilization (Sensui and Morisawa, 1996). Heparin prevented $[Ca^{2+}]_i$ transients and extrusion of the second polar body. Postactivation Ca^{2+} waves may be required for extrusion of the second polar body in another ascidian *P. mammillata* (McDougall and Sardet, 1995). Therefore, $[Ca^{2+}]_i$ transients after fertilization may drive metaphase–anaphase transition in meiosis. It is probable that $[Ca^{2+}]_i$ transients in Series I drive metaphase I to metaphase II, and that Series II drives metaphase II to completion of meiosis in the egg.

Because the maturation promoting factor (MPF) has an essential role in regulating metaphase–anaphase transition in the cell cycle, it is suggested that IP_3R-mediated Ca^{2+} release regulates MPF activity. It is clear that $[Ca^{2+}]_i$ participates in inactivation of MPF, and calmodulin-dependent protein kinase II (CaM kinase II) is thought to mediate the inactivation of MPF (Lorca *et al.*, 1993). However, the relationship between CaM kinase II and activity of MPF is unknown. On the other hand, correlation between Ca^{2+} and the activity of 26S proteasome, which decreases MPF activity was noted in the ascidian *Halocynthia roretzi* (Kawahara and Yokosawa, 1994). A role for Ca^{2+} in the context of the cell cycle will need to be investigated in *in vivo* studies.

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