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Effects of the Phosphatase Inhibitors, Okadaic Acid, ATPγS, and Calyculin A on the Dividing Sand Dollar egg

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ABSTRACT. **The effects of the phosphatase inhibitors, okadaic acid (OA), adenosine 5'-O-(3-thiotriphosphate) (ATPγS), and calyculin A (CL-A) on anaphase chromosome movement, cytokinesis, and cytoskeletal structures at cell division were examined by being microinjected into mitotic sand dollar eggs. When OA was injected, chromosome movement was inhibited and, moreover, chromosomes were ejected from the polar regions of the mitotic apparatus. By immunofluorescence, microtubules were observed to be severed in the OA-injected eggs, causing the smooth cell surface to be changed to an irregular surface. When ATPγS and CL-A were injected, the effect on cell shape was remarkable: In dividing eggs, furrowing stopped within several seconds after injection, small blebs appeared on the cell surface and became large, spherical or dumbbell cell shapes then changed to irregular forms, and subsequently cytoplasmic flow occurred. Microfilament detection revealed that actin accumulation in the cortex, which was not limited to the furrow cortex, occurred shortly after injection. Cortical accumulation of actin is thought to induce force generation and random cortical contraction, and accordingly to result in bleb extrusion from the cortex. Consequently, the phosphatase inhibitors inhibited the transition from mitosis to interphase by mediating cortical accumulation of actin filaments and/or fragmentation of microtubules.**

Key words: actin/chromosome movement/cleavage furrow/microtubule/phosphatase inhibitor/sand dollar egg

The mitotic cell cycle is controlled by reversible protein phosphorylation. The extent of protein phosphorylation, which reflects the relative activities of protein kinases and phosphatases, oscillates in parallel with the cell cycle. In general, progression from interphase into mitosis is characterized by specific phosphorylation events and progression from mitosis to interphase is characterized by a series of dephosphorylation events (Mabuchi, 1993; Vandre and Borisy, 1989). Morphologically, the transition from mitosis back to interphase is initiated upon anaphase onset and finished at the end of cytokinesis.

In order to understand the role of protein phosphatases, which dephosphorylate phosphoproteins in the cell cycle, phosphatase inhibitors have been used. Adenosine 5'-O-(3-thiotriphosphate) (ATPγS) produces thiophosphorylated proteins which are resistant to phosphatases. In this sense ATPγS is an inhibitor for all phosphatases including protein phosphatases and has mainly been used in vitro experiments (Lohka *et al*., 1987; Wordeman *et al*., 1989). ATPγS was also used in living cells by microinjection for investigating exocytosis during fertilization (Hamaguchi, 1989; Whalley *et al*., 1991). Calyculin A (CL-A) and okadaic acid (OA) are known as specific inhibitors for protein phosphatase 1 and 2A (Bialojan and Takai, 1988; Cohen 1989; Ishihara *et al*., 1989).

It has frequently been reported that OA promotes mitosis-like phenomena or induces premature mitosis (Gavin *et al*., 1991; Gliskman *et al*., 1992; Goris *et al*., 1989; Patel and Whitaker, 1991; Picard *et al*., 1989, 1991; Rime and Ozon, 1990; Thyberg and Moskalewski, 1992; Yamashita *et al*., 1990). CL-A was found to induce chromosome condensation in unfertilized sea urchin eggs (Tosuji *et al*., 1992). On the other hand, it is unclear whether or not phosphatase inhibitors block the transition from mitosis to interphase, although Vandre and Wills (1992) reported that OA inhibited

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Abbreviations: ATPγS, adenosine 5'-O-(3-thiotriphosphate); CL-A, calyculin A; DIC, differential interference-contrast; DMSO, dimethylsulfoxide; MOPS, 3-morpholinopropanesulfonic acid; OA, okadaic acid.

anaphase transition of mammalian cultured cells. Furthermore, it has been reported that OA was not effective in sea urchin eggs (Asano and Mabuchi, 2001; Tosuji *et al*., 1992). Microinjection of this inhibitor may be useful to investigate its effects on cell motility in sea urchin eggs because microinjection has the merit of introducing an inhibitor unable to cross the cell membrane.

In this study, the effects of the phosphatase inhibitor, OA on cell division, especially on anaphase chromosome movement and cleavage furrowing, were investigated by being microinjected into fertilized sand dollar eggs. Moreover, the effects of the other phosphatase inhibitors, ATPγS and CL-A, were investigated in comparison to those of OA. The effects of OA, ATPγS, and CL-A on cytoskeletal structures at cell division were also examined.

Materials and Methods

Biological materials

Gametes of the sand dollar, *Clypeaster japonicus*, were obtained by injection of 1–10 mM acetylcholine in sea water into the body cavity. Eggs were washed three times by artificial sea water (Jamarin-U, Jamarin Laboratory, Osaka), kept at 15°C. Sperm were collected as "dry sperm," kept at 4°C in a refrigerator and diluted just before use. For chromosome observation in the living egg, the unfertilized egg was treated for 5 min with Hoechst 33342 at 5 μM before fertilization, washed two times with artificial sea water, and then fertilized. Fertilized eggs were deprived of both the fertilization envelope and hyaline layer by being treated for 1.5 min with a 1 M urea solution shortly after insemination and incubated in Ca-free sea water (Ca-free Jamarin-U, Jamarin Lab., Osaka) at 25–27°C.

Microinjection

For injection the eggs were transferred to artificial sea water and introduced to the injection chamber (Kiehart, 1981). In the case of staining after microinjection, the eggs were attached to a polylysine-coated coverslip (Mazia *et al*., 1975), incubated in artificial sea water, and injected with inhibitors by the method described by Hamaguchi (1998). As the mean egg diameter was 115 μm, the egg volume was estimated as 800 pl. CL-A and OA were dissolved at 1.0–2.0 mg/ml (1.0–2.0 mM) and 0.067–2.0 mg/ml (0.081–2.5 mM) in dimethylsulfoxide (DMSO), respectively, in order that the injected volume was less than 1% of the egg volume. The injection of DMSO at 1% of the egg volume did not show any effect on the development of the egg. ATPγS was dissolved at 2–50 mM in 50 mM 3-morpholinopropanesulfonic acid (MOPS) solution at pH 7.0 and its injected volume was less than 50 pl. These eggs were directly or after staining used for morphological and birefringence examinations.

Fluorescence staining

Indirect immunofluorescence was carried out using an anti-tubulin antibody according to Hamaguchi (1998). Several minutes after the injection of OA the eggs, fertilized and attached to the polylysine-coated coverslip, were permeabilized with a microtubule-stabilizing solution (5 mM EGTA, 20 mM 1,4-piperazinediethanesulfonic acid, 2 mM $MgCl_2$, 1 M glycerol, pH was adjusted to 7.0 by KOH) supplemented with surfactants of 1% Nonidet P-40 and 0.2% 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonic acid and extracted for 1 hr in the solution. These eggs were fixed for 20 min with 0.3 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and then for 20 min with 1.8% formaldehyde in the MT-stabilizing solution without surfactants. They were stained with the anti-tubulin antibody, DM1A (Amersham, England) for MT observation and then stained with 4',6-diamidino-2-phenylindole at 0.5 μM for chromosome observation.

Fluorescence labeling of microfilaments was carried out according to Mabuchi (1994) with slight modification. The glucose F-buffer contained 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM MOPS (pH 7.4), and 0.6 M glucose. Fertilized eggs were fixed for 20–30 min with glucose F-buffer supplemented with 1.8% formaldehyde, 0.1% glutaraldehyde, and 0.05% Nonidet P-40 and then extracted for 30 min with glucose F-buffer supplemented with 0.5% Nonidet P-40. They were stained for 30 min with rhodamine-phalloidin or AlexaFluor 488-phalloidin for actin filament observation. Rhodamine-phalloidin and AlexaFluor 488-phalloidin solution dissolved in methanol (Molecular Probes, Inc., Eugene, OR) were diluted 100 times in glucose F-buffer supplemented with 1% β-mercaptoethanol before use.

Observation

The living egg was observed with a fluorescence and polarization microscope using a Brace-Kohler compensator (retardation value of 31.4 nm, Nikon Inc., Tokyo). Images were taken directly with a microscope camera (UFX-DX, Nikon Inc.), or images which were displayed on a video monitor (C-1846-01, Hamamatsu Photonics, Hamamatsu, Japan) were taken indirectly. The images were recorded with a video cassette recorder (MacLord Hi-Fi GT4, National, Tokyo) using a video camera (WV-1550, National) equipped with a Nikon zoom lens after being processed with an image processor (Image Sigma II, Avionics Co. Ltd., Tokyo). The eggs stained for immunofluorescence or fluorescence labeling of microfilaments were observed with a microscope equipped with fluorescence and differential interference-contrast (DIC) optics and were taken on a negative film with a microscope camera, or were observed with a fluorescence microscope equipped with a confocal fluorescence apparatus (INSIGHTPLUS, Meridian Instruments Inc., Okemos, MI) and taken with a CCD camera (C2400-75I, Hamamatsu Photonics) using a computer through an image processor (Argus 20, Hamamatsu Photonics).

The fluorescence intensity of the injected egg was calculated as follows. The exposure times of the whole egg and background were measured with a microscope camera (UFX-DX), the reciprocals of the exposure times of several eggs were averaged after background subtraction, and the mean reciprocal was the fluores-

cence intensity. The fluorescence intensity of the injected egg was compared to that of the uninjected egg in each experiment.

Results

General remarks

In *Clypeaster japonicus* the duration from fertilization to the midstage of first cleavage when the egg divides halfway averaged 65 min at 25–27°C. In order not to inhibit the cell cycle before nuclear envelope breakdown, injection was carried out from 40 min (just before nuclear envelope breakdown) to 55 min (the end of metaphase) after insemination and the result was judged by whether or not anaphase chromosome movement and/or cytokinesis happened in the injected eggs by 75 min after insemination. Percentage of the inhibited eggs to total injected ones was designated as % inhibition. Cytokinesis, which depends on anaphase, started 6 min later after the beginning of anaphase, and so the duration when the egg cytoplasm was exposed to the inhibitors was longer by 6 min for cytokinesis than for anaphase chromosome movement. Therefore, it is natural that the threshold concentration was usually lower for furrowing inhibition rather than for the inhibition of chromosome movement, although the molecular mechanisms for chromosome movement and furrowing are quite different.

In order to investigate the effects of phosphatase inhibitors on chromosome movement or furrowing, the injection at high dose was carried out even during anaphase chromosome movement or cytokinesis. Morphological changes induced in cytoskeletal structures were analyzed shortly after injecting inhibitors.

Okadaic acid injection

OA was injected into sand dollar eggs at a final concentration of 2 μM or less. The inhibitory effect on cell division occurred in a dose-dependent manner (Fig. 1a). The threshold concentration is designated in this study as the concentration when the inhibitory effect was detected in half of the eggs injected with the inhibitor. The threshold concentration of OA for chromosome movement inhibition was 0.5–1 μM and that for cleavage inhibition was about 0.2–0.5 μM. The inhibitory effect on chromosome movement occurred (Figs. 2 and 3) when the egg was injected at metaphase. Thin fibers from chromosomes were pulled toward the poles in the egg injected with OA at metaphase, which indicates that anaphase started in this egg, but chromatid separation might be inhibited (Figs. 3d and d'). The nuclear envelope did not form and the cluster of chromosomes moved away from the mitotic apparatus. Effects on microtubules such as a decrease in birefringence of the mitotic apparatus were detected (Fig. 3). Moreover, even when the egg was injected at anaphase, chromosome movement was inhibited; chromosomes moved randomly. Chromosomes, which had moved

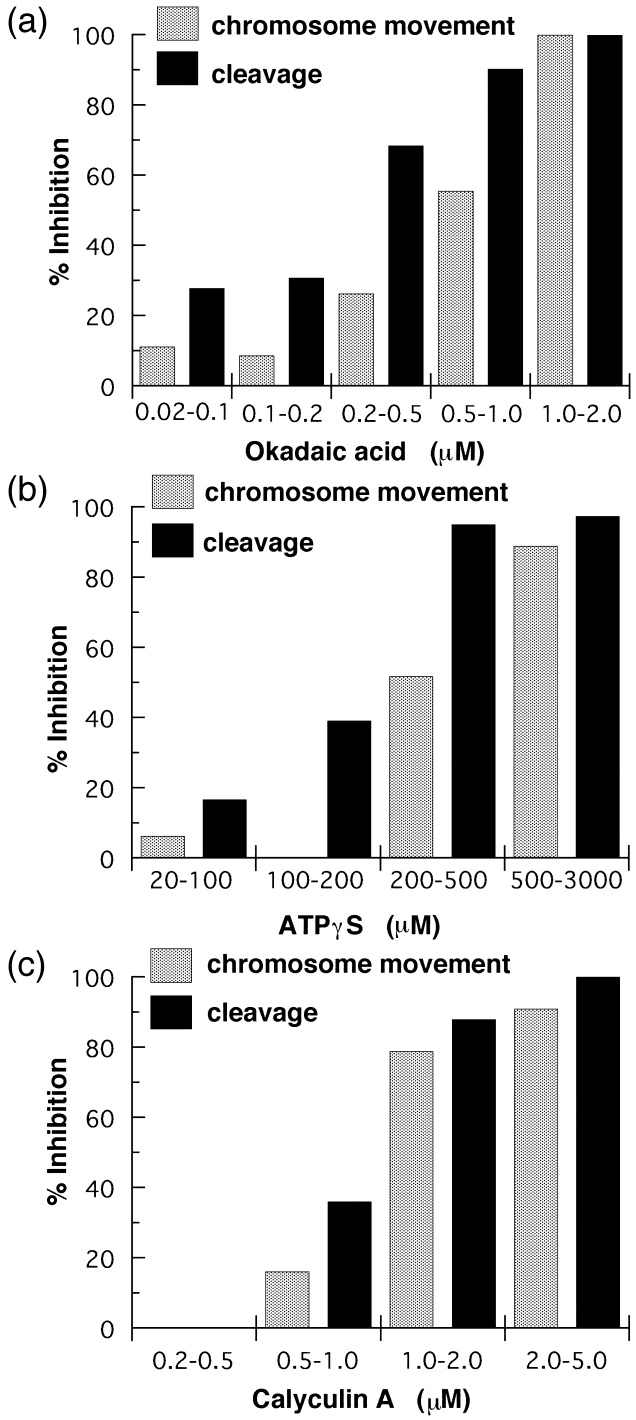

Fig. 1. Inhibition of chromosome movement and first cleavage in the eggs injected with phosphatase inhibitors. (a) Injection of OA. Each column contains 9–52 samples. (b) Injection of ATPγS. Each column contains 12–38 samples. (c) Injection of CL-A. Each column contains 14–33 samples. Abscissa: final concentration of a phosphatase inhibitor in μM. Ordinate: % inhibition of chromosome movement or cleavage.

once to the spindle poles, returned backward to the metaphase plate, and accordingly the chromosomes distributed randomly between the spindle poles (Fig. 4). In order to investigate the effects of OA on microtubules, the eggs injected with OA were stained with anti-tubulin antibody by indirect immunofluorescence. The shape of the mitotic apparatus became quite different from that of control eggs. The spindle became thin at the interzone and microtubules, and especially those of asters were fragmented and dispersed all over the egg cytoplasm (Fig. 5).

The remarkable effects on the cell shape were observed by injection of OA (Fig. 6). In order to investigate the effect of OA on microfilaments, the egg injected with OA was stained with fluorescently labeled phalloidin. The cortex became fluorescently bright in comparison to the cortex of control eggs (Fig. 7a and b). The fluorescence intensity of the whole egg injected at the final concentration of 1.8–1.9 μM increased to 2.1±0.2 times (sample no. 18) higher than that of a control egg at 6–11 min after the injection. The accumulation became even all over the cortex and accordingly actin accumulation in the furrow became less prominent (Fig. 7c and d).

OA was not effective up to 10 μM when it was applied to the sand dollar eggs by being dissolved in external sea water. This result suggests that OA cannot permeate the cell membrane of sand dollar eggs.

ATPγS injection

ATPγS was injected into sand dollar eggs at the final concentration of 3 mM or less. The inhibitory effect on cell division occurred in a dose-dependent manner (Fig. 1b). The threshold concentration of ATPγS for chromosome movement inhibition was 200–500 μM and that for cleavage inhibition was 100–200 μM. The remarkable effect was to change the shape of the egg. To begin with, bleb formation occurred and the spherical shape of cells changed into an irregular one (Fig. 8). Effects on microtubules such as a decrease in birefringence of the mitotic apparatus were not detected (Figs. 8a–c); however, the mitotic apparatus was disintegrated by means of cytoplasmic streaming due to the cell shape change. Even when the egg was furrowing, cleavage inhibition occurred; furrowing stopped without regressing, blebs formed and became large, and the cell shape changed irregularly as if the egg were an amoeba (Fig. 9).

In order to investigate the effect of ATPγS on microfilaments, the egg injected with ATPγS was stained with fluorescently labeled phalloidin. The cortex became fluorescently bright in comparison to the cortex of control eggs (Fig. 7e and f). The fluorescence intensity of the whole egg injected at the final concentration of 1.2–1.3 mM increased to 4.4±1.4 (sample no. 26) times higher than that of a control egg at 5-8 min after the injection. The accumulation be-

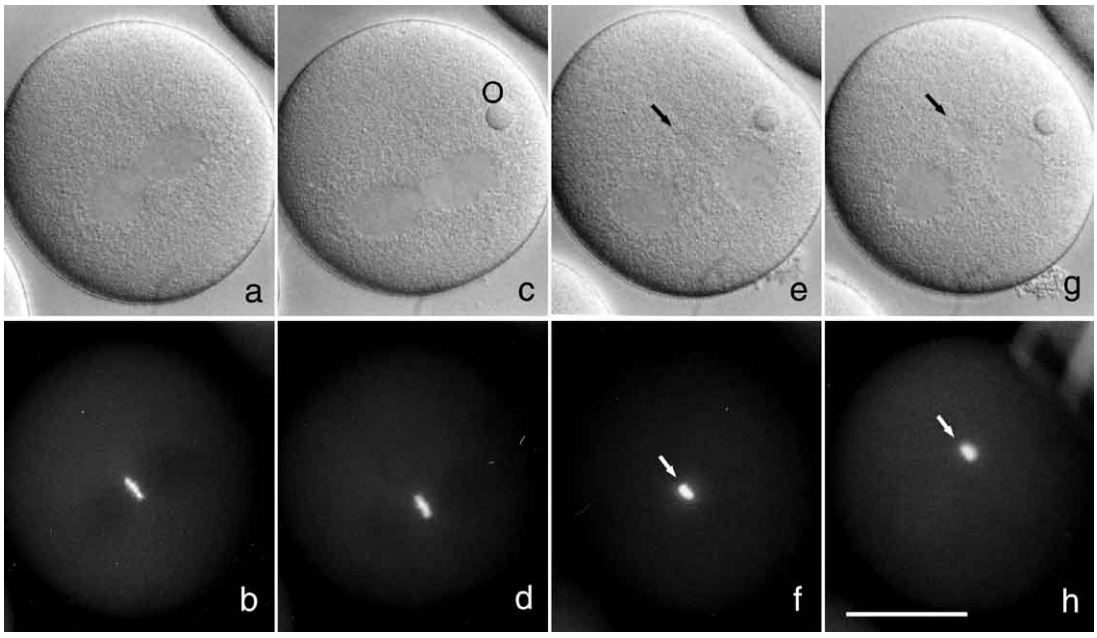

Fig. 2. Effects of OA injection at metaphase on chromosome movement. (a), (b) Before injection at metaphase. (c), (d) Shortly after injection. O is an oil drop injected simultaneously with the injectate. (e), (f) Chromosomes did not move but formed an aggregate. (g), (h) Aggregated chromosomes (arrow) were ejected from the asters. The egg was injected with OA at the final concentration of 1.5 μM at 52 min after fertilization. The micrographs in the upper row are DIC ones and those in the lower row are fluorescence ones. (a) and (b), (c) and (d), (e) and (f), and (g) and (h) were taken –1, 1, 17, and 40 min after injection, respectively. The scale bar is 50 μm.

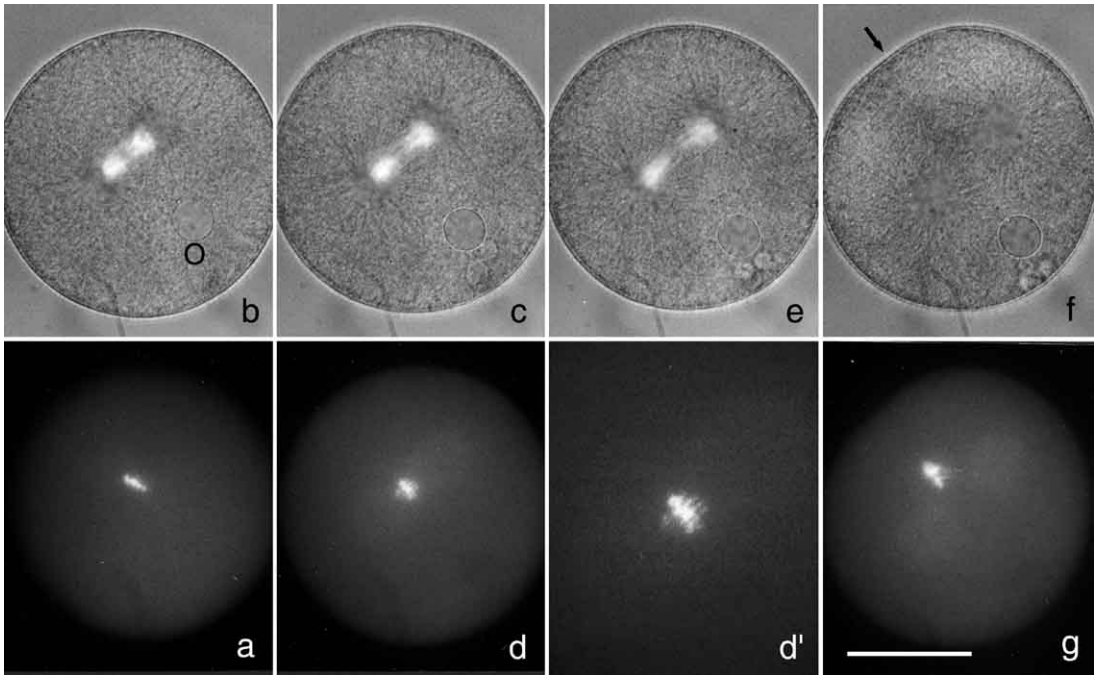

Fig. 3. Effects of OA injection at metaphase on spindle configuration and chromosome movement. (a) Before injection. (a) is set beneath (b) in order to align the polarization micrographs in the upper row. (b) Shortly after injection at metaphase. O is an oil drop injected simultaneously with the injectate. (c), (d), (d') Chromosomes did not move but formed an aggregate. Slender fluorescent threads were pulled from the aggregate. The spindle became slender. The image of (d') is the central region of (d) at two times magnification. Fluorescent threads pulled toward the spindle poles were clearly demonstrated. (e) The spindle became long and slender. (f), (g) Aggregated chromosomes were ejected from the asters. Birefringence was not detected anymore. The furrow (arrow) was forming at the upper side of the egg. The egg was injected with OA at the final concentration of 1.5 μM at 54 min after fertilization. The micrographs in the upper row are polarization ones and those in the lower row are fluorescence ones. (a), (b), (c), (d), (e), (f), and (g) were taken –1 min, 20 sec, 2 min, 2 min 20 sec, 3 min, 6 min 30 sec, and 9 min after injection, respectively. The scale bar is 50 μm.

came even all over the cortex and accordingly actin accumulation in the furrow became less prominent (Fig. 7g and h).

Calyculin A injection

CL-A was injected into sand dollar eggs at a final concentration of 5 μM or less. The inhibitory effect on cell division occurred in a dose-dependent manner (Fig. 1c). The threshold concentration of CL-A for chromosome movement inhibition was 1–2 μM and that for cleavage inhibition was about 1 μM. A remarkable effect was observed on cell shape in the manner similar to ATPγS (Fig. 10). Even when the egg was furrowing, division inhibition occurred by microinjection of CL-A as well. Effects on microtubules such as a decrease in birefringence of the mitotic apparatus were not detected (not shown).

In order to investigate the effect of CL-A on microfilaments, the egg injected with CL-A was stained with fluorescently labeled phalloidin. The cortex became fluorescently bright in comparison to the cortex of control eggs similarly to ATPγS (Figs. 7i and j). The fluorescence intensity of the whole injected egg increased to about 3 times higher than that of a control egg as measured by the photometer of the microscope camera, although the fluorescence intensity of the injected region increased heterogeneously as shown in Figs. 7j and l.

Discussion

Difference of the inhibitory effects among ATPγS, CL-A, and OA

In this study the effects of ATPγS, CL-A, and OA on cell division, especially on motility at division, were investigated by being microinjected at mitosis, as summarized in Table I. They inhibited cell division in a dose-dependent manner. Their threshold concentrations were different; ATPγS might be the least effective among them, which is natural because ATP, which may be used competently by protein kinases, is rich in the cytoplasm. The threshold concentrations of CL-A for chromosome movement and cleavage furrowing were higher than those for OA, although CL-A is known to be more effective against phosphatase 1 than OA but as effective against phosphatase 2A as OA. CL-A is also known to

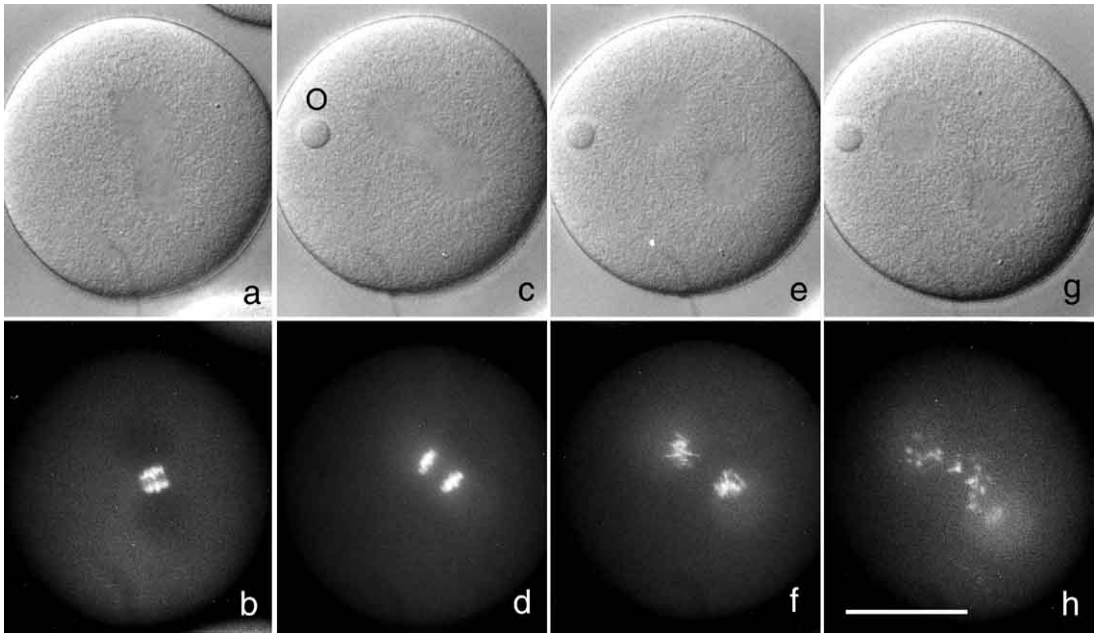

Fig. 4. Effects of OA injection at anaphase on chromosome movement. (a), (b) Before injection at anaphase. (c), (d) Shortly after injection chromosomes were still moving. O is an oil drop injected simultaneously with the injectate. (e), (f) The chromosomes reached poles but did not align. (g), (h) The egg shape elongated slightly. The chromosomes randomly moved backward to the equator. The egg was injected with OA at the final concentration of 1.5 μM at 55 min after fertilization. The micrographs in the upper row are DIC ones and those in the lower row are fluorescence ones. (a) and (b), (c) and (d), (e) and (f), and (g) and (h) were taken –1, 1, 5, and 13 min after injection, respectively. The scale bar is 50 μm.

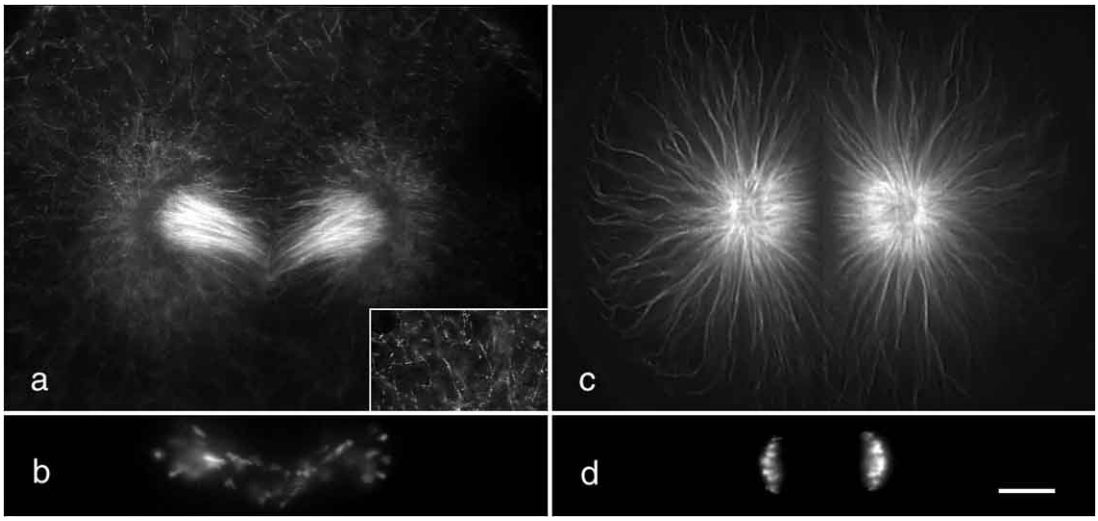

Fig. 5. Immunofluorescence micrographs stained with anti-tubulin antibody. (a), (b) The region of the mitotic apparatus in the egg injected with OA at the final concentration of 1.7 μM at 59 min after insemination (anaphase) and extracted 7 min later. Inset in (a) is printed at excess exposure and shows severed microtubules in the peripheral region of the injected egg. (c), (d) The region of the mitotic apparatus in a control egg at late anaphase. (a), (c) are fluorescence micrographs showing microtubules and (b), (d) are fluorescence micrographs showing chromosomes. The scale bar is 10 μm.

be effective by being applied in a culture medium, which means that it might diffuse quickly from the cytoplasm to the medium when it is introduced into the egg cytoplasm by microinjection. On the other hand, OA is not effective by being applied in a cultured sea water to sea urchin eggs (Asano and Mabuchi, 2001; Tosuji *et al*., 1992; present study), which indicates that OA may not permeate the egg membrane.

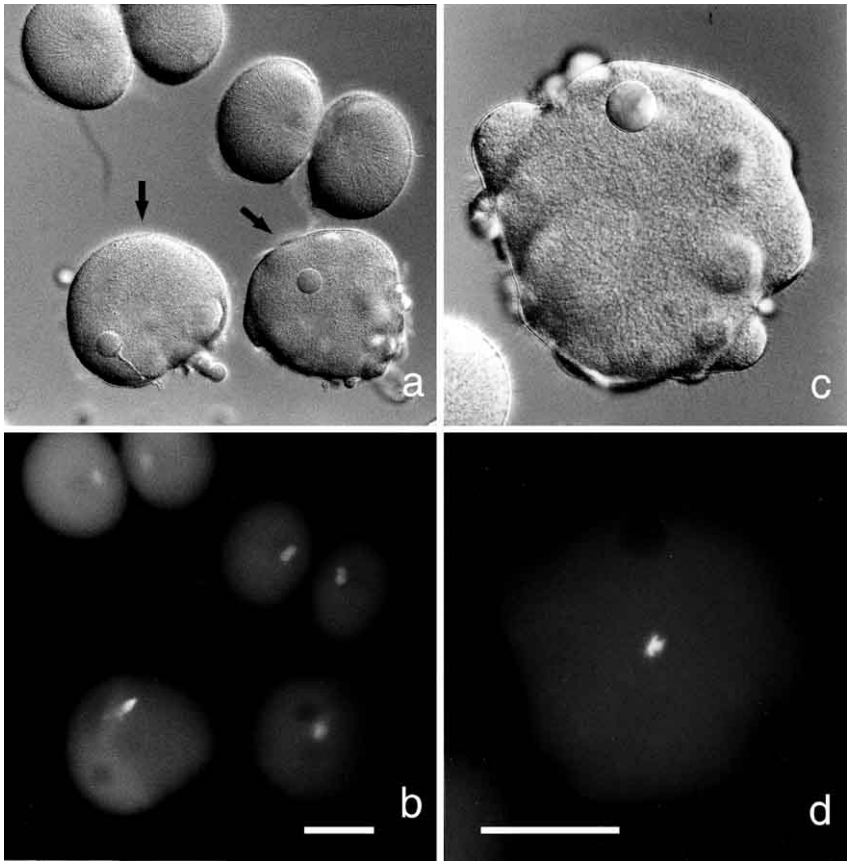

Fig. 6. Effects of OA injection on the cell shape. (a), (b) The eggs (arrows) were injected with OA at the final concentrations of 0.45 μM at 49 and 50 min after fertilization (metaphase). They were taken 65 min after fertilization. The surface of the injected eggs became irregular. Chromosome movement did not occur in the injected eggs, whereas it occurred in control eggs, which had already divided. (c), (d) The egg was injected with OA at the final concentration of 0.53 μM 54 min after fertilization (prometaphase) and taken 74 min after fertilization. Chromosome movement did not occur. (a), (c) are DIC micrographs and (b), (d) are fluorescence micrographs. The scale bar is 50 μm.

The effects of ATPγS, CL-A, and OA on cell motility during cell division were somewhat different. ATPγS and CL-A injection induced similar effects on the cell cortex. CL-A is a specific inhibitor for protein phosphatase 1 and 2A at the same level of efficiency and, according to the similarity of the effects on the cell cortex, ATPγS, as well as CL-A, apparently inhibited phosphatase 1 and 2A during cell division. Protein phosphatase 1 but not 2A modulates actin filament integrity and myosin light chain phosphorylation in nonmuscle cells (Fernandez *et al*., 1990); therefore, the effects of ATPγS and CL-A may be caused mainly by mediating the inhibition of phosphatase 1. On the other hand, OA injection induced inhibitory effects on chromosome movement adding to the other effects similar to ATPγS and CL-A. Because OA is known to be much more effective against phosphatase 2A than against phosphatase 1 (Bialojan and Takai, 1988; Ishihara *et al*., 1989), the effects of OA may be caused mainly by mediating the inhibition of phosphatase 2A. It is also reported that phosphatase 2A possibly was involved in anaphase transition (Vandre and Wills, 1992).

Inhibition of chromosome movement

When OA was injected, chromosomes did not only stop moving forward to the spindle poles, but also moved backward to the equatorial plane and then chromosomes were distributed randomly between the two spindle poles. We found by polarization microscopy that the spindle became slender and its birefringence became weak and by immunofluorescence that microtubules were severed. Severing protein was reported in sea urchin eggs (McNally and Vale, 1993; Quarmby and Lohret, 1999), although the effect of OA on the severing activity of the protein has not yet been investigated. After a while, these fragmented microtubules and then the mitotic spindle might disappear. It has been reported that OA induced the disappearance of the mitotic spindle (Gavin *et al*., 1991; Picard *et al*., 1989; Rime and Ozon, 1990) or that the spindle was disrupted (Vandre and

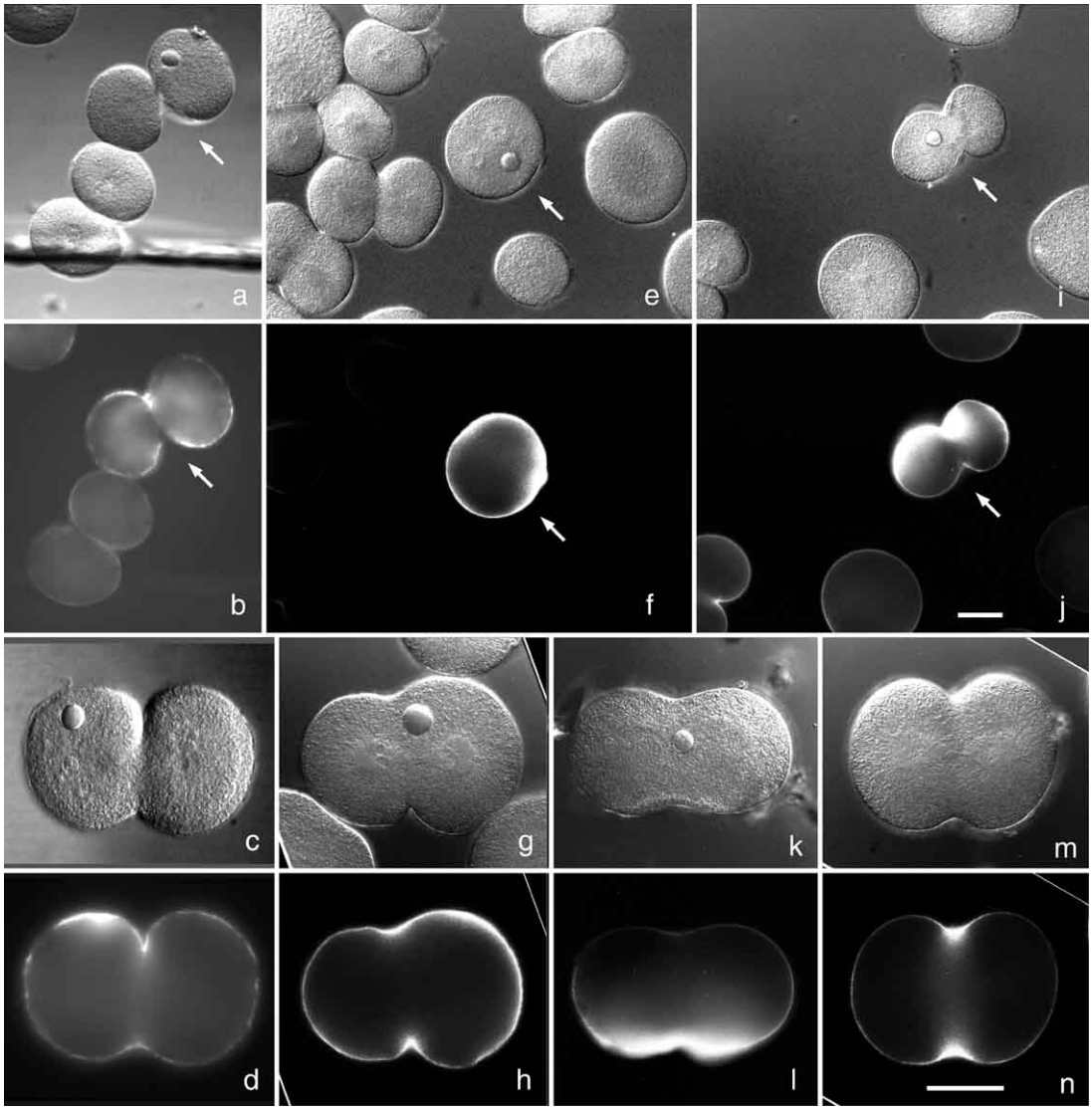

Fig. 7. Microfilament staining after injection of OA, ATPγS, and CL-A. The egg in (a) and (b) was injected with OA at the final concentration of 1.8 μM at 53 min after fertilization and fixed 6 min later. The egg in (c) and (d) was injected with OA at the final concentration of 1.9 μM at 56 min after fertilization and fixed 7 min later. The egg in (e) and (f) was injected with ATPγS at the final concentration of 0.94 mM at 59 min after fertilization and fixed 7 min later. The egg in (g) and (h) was injected with ATPγS at the final concentration of 0.48 mM at 59 min after fertilization and fixed 8 min later. The egg in (1) and (j) was injected with CL-A at the final concentration of 10 μM at 63 min after fertilization and fixed 5 min later. The egg in (k) and (l) was injected with CL-A at the final concentration of 10 μM at 61 min after fertilization and fixed 7 min later. (m) and (n) are controls. In order to show cortical actin, the egg in (n) was taken at automatic exposure, although the fluorescence intensity of the egg was much lower than those of the eggs in (d), (h), and (l). Arrows indicate the injected eggs. (a), (c), (e), (g), (i), (k), and (m) are DIC micrographs and (b), (d), (f), (h), (j), (l) and (n) are fluorescence micrographs. The scale bar in (j) is 50 μm for (a), (b), (e), (f), (i) and (j). The scale bar in (n) is 50 μm for (c), (d), (g), (h), (k), (l), (m) and (n).

Wills, 1992). On the other hand, it was reported that mitotic asters instead of the asters at interphase appeared (Thyberg and Moskalewski, 1992; Yamashita *et al*., 1990).

Chromosomes might be ejected from the polar regions of the mitotic apparatus or ejected backward on the midway to the poles because there still remained many microtubules around the poles in the slender spindle, even though kinetochore microtubules were severed. Chromosome ejection were reported by polar microtubules and/or by motor proteins (Skibbens *et al*., 1993; Walczak *et al*., 1998), which might be activated by phosphatase inhibitors.

When ATPγS and CL-A were injected, chromosome movement was inhibited but they neither stopped the movement of chromosome nor decreased the spindle birefringence. At high-dose injections of these inhibitors, we could not detect their effects on microtubules because the

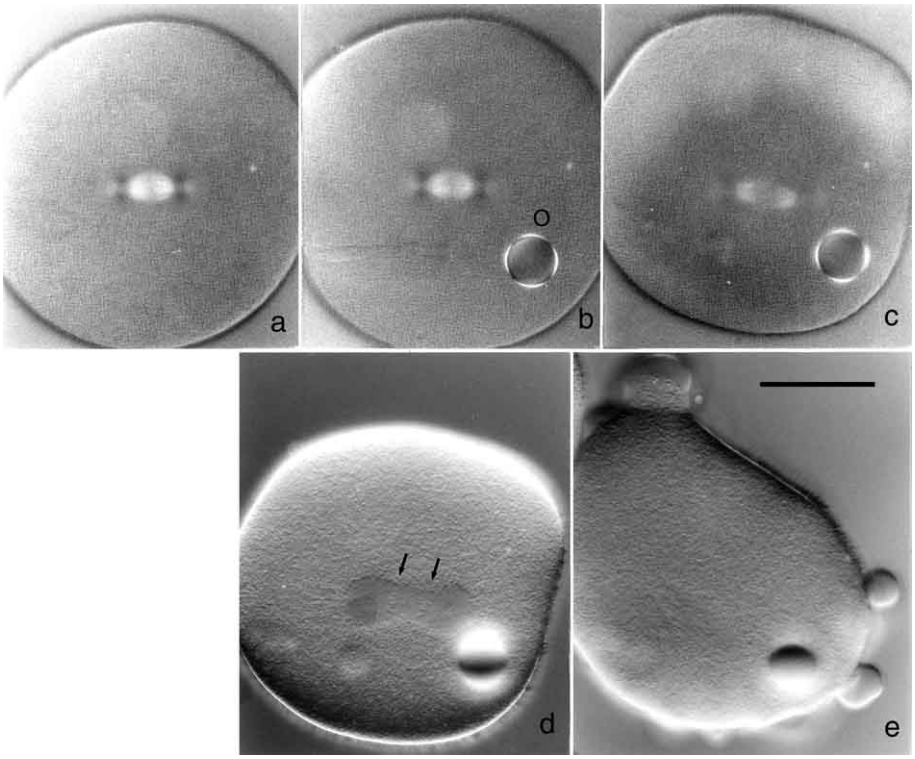

Fig. 8. Effects of ATPγS injection on spindle birefringence and cell shape. (a) 20 sec before injection at metaphase. (b) 20 sec after injection. The egg was injected with ATPγS at the final concentration of 1.0 mM at 49 min after fertilization. O is an oil drop injected simultaneously with the injectate. (c) 4 min 10 sec after injection the cell shape became somewhat irregular. (d) 4 min 40 sec after injection. The cell shape became irregular. Arrows indicate two groups of chromosomes. (e) 28 min after injection. Some blebs were extruded from the surface of the egg. (a), (b), and (c) are polarization micrographs, and (d) and (e) are DIC micrographs. The scale bar is 50 μm.

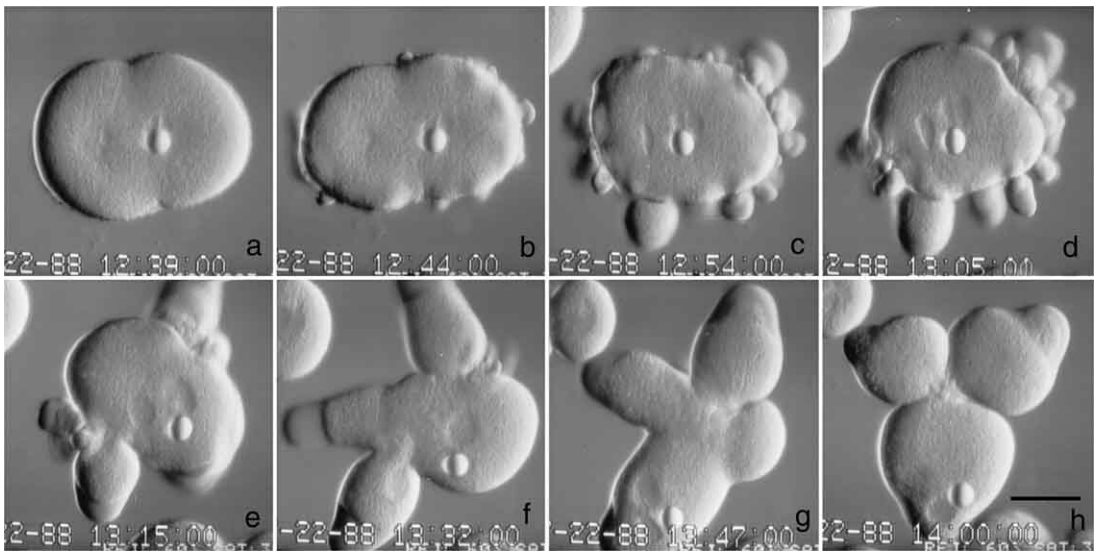

Fig. 9. Effects of ATPγS injection during cleavage. The egg was injected with ATPγS at the final concentration of 0.72 mM at 65 min after fertilization. By DIC microscopy, (a), (b), (c), (d), (e), (f), (g), and (h) were taken 7, 12, 22, 33, 43, 60, 75, and 88 min after the injection, respectively. The scale bar is 50 μm.

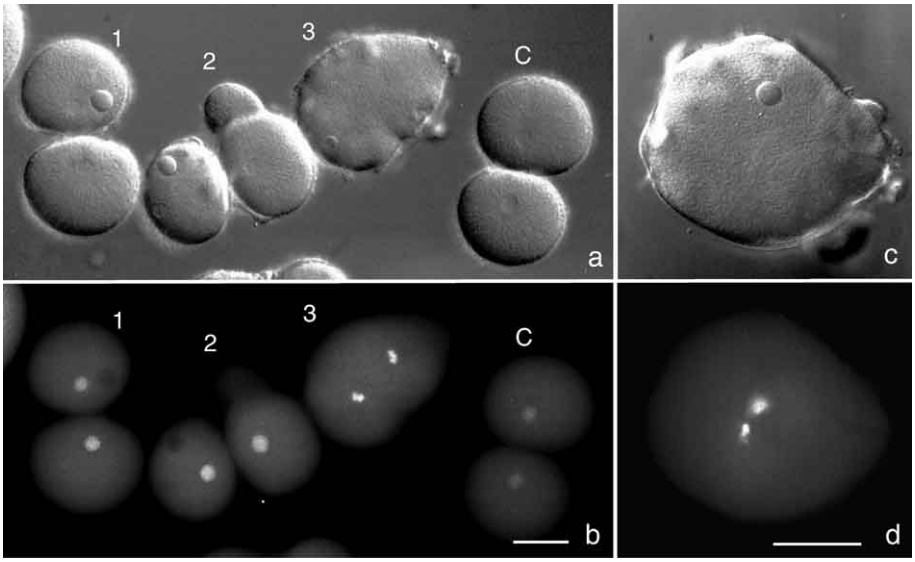

Fig. 10. Effects of CL-A injection on the cell shape. (a), (b) The eggs labeled with (1), (2), and (3) were injected with CL-A at the final concentrations of 0.35, 0.90, and 1.7 μM at 49, 50, and 51 min after fertilization (metaphase), respectively, and the egg labeled with C was a control. They were taken 70 min after fertilization. The injected eggs (1) and (2) divided, although the surface of the egg (2) became irregular. The injected egg (3) did not divide and its surface became quite irregular, although chromosome movement occurred. (c), (d) The egg was injected with CL-A at the final concentration of 1.8 μM at 46 min after fertilization (prometaphase) and taken 67 min after fertilization. Chromosome movement did not occur, although the chromosomes aggregated into two clusters. (a), (c) are micrographs and (b), (d) are fluorescence micrographs. The scale bar is 50 μm.

Table I. DIFFERENCE OF THE INHIBITORY EFFECTS AMONG THE PHOSPHATASE INHIBITORS, OKADAIC ACID (OA), ADENOSINE 5'-O-(3-THIOTRIPHOSPHATE) (ATPγS), AND CALYCULIN A (CL-A)

Inhibitor	Threshold conc. for inhibition of chromosome movement and cleavage	Effect on actin filaments	Effect on microtubules
OA	0.2–1 μM	++	+++
ATPγS	0.1–0.5 mM	+++	±
CL-A	1–2 μM	+++	±, +[1)]

1) ±: present paper, +: Hosoya *et al.* (1993)

mitotic apparatus was disrupted by changes in cell morphology. Hosoya *et al*. (1993) reported slight effects on the mitotic spindle in CL-A treated cells.

Microfilament accumulation in the cell cortex by microinjecting the inhibitors

When OA, ATPγS, and CL-A were injected, microfilament detection revealed that actin accumulation in the cortex occurred shortly after injection and resulted in uniform distribution of actin in the cortex. Phosphatase inhibitors are reported to induce myosin phosphorylation, which might induce activation of myosin ATPase (Fernandez *et al*., 1990). Accordingly, excess forces were generated in the cortex and then those forces induced random cortical contraction, which resulted in changes egg morphology. The effects of CL-A on the cell cortex were reported by means of incubating the eggs with the inhibitor (Asano and Mabuchi, 2001; Mabuchi *et al*., 1996; Tosuji *et al*., 1992, 2000).

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