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Calyculin A induces contractile ring-like apparatus formation and condensation of chromosomes in unfertilized sea urchin eggs

(phosphatase inhibitor/microfilaments/cell division/histone kinase)

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ABSTRACT **Calyculin A, a protein phosphatase inhibitor, induced cleavage-like morphological change in unfertilized sea urchin eggs. A contractile ring-like apparatus containing both filamentous actin and myosin was formed in the cleavage furrow. Wheat germ agglutinin receptors were also found in the same region. The eggs did not develop further after constriction of the ring. No aster-like microtubular structure was found in the calyculin A-treated eggs. The cleavage was not inhibited by the antimicrotubule drug griseofulvin. Calyculin A also increased histone H1 kinase activity and induced chromosome condensation. These changes also occurred in the presence of emetine (an inhibitor of protein synthesis) and aphidicolin (an inhibitor of DNA synthesis). It is suggested that calyculin A induced these changes in the sea urchin eggs by inhibiting the activity of protein phosphatase 1.**

The motive force of cleavage in animal cells is generated by the interaction between actin filaments and myosin in the contractile ring (CR) or the contractile arc (1, 2). It has been proposed (3) that induction of the cleavage furrow is mediated by an as-yet-uncharacterized substance that is transported from the mitotic apparatus to the cortical layer via astral microtubules at a specific stage of cell division. The CR forms rapidly but reduces its volume during contraction and disappears soon after cytokinesis (4). The mechanisms that induce this sequence of events and the nature of the signal that stimulates the furrow formation have not yet been elucidated. One interesting approach to this problem is to identify substances that induce cleavage or a specific stage in the cleavage process.

Calyculin A (CL-A), a tumor-promoting substance isolated from the marine sponge *Discodermia calyx* (5), is a specific inhibitor of protein phosphatase 1 (PP1) (IC_{50} = 0.5–1 nM) and protein phosphatase 2A (PP2A) (IC_{50} = 2 nM) in rabbit skeletal muscle (6). It has been shown to induce oocyte maturation in starfish (7) and stimulate contraction of smooth muscle (6), effects also induced by the phosphatase inhibitor okadaic acid (OA). We report here that CL-A induces a CR-like apparatus in unfertilized sea urchin eggs. The simultaneous condensation of chromosomes was also observed.

MATERIALS AND METHODS

Materials. The species of sea urchins used in this study were *Anthocidaris crassispina, Clypeaster japonicus, Diadema savignyi, Diadema setosum, Echinometra mathaei, Echinostrephus aciculatus, Hemicentrotus pulcherrimus, Pseudocentrotus depressus, Scaphechinus mirabilis, Stomopneustes variolaris, Strongylocentrotus nudus, Temnopleurus hardwicki, Toxopneustes pileolus*, and *Tripneustes gratilla*. Mature eggs were obtained by injecting 0.1 M acetylcholine chloride into the body cavity. CL-A was isolated from the marine sponge *Discodermia calyx*, as described (5).

Fluorescence Microscopy. Eggs were allowed to settle onto protamine-coated glass slides and were fixed with 5% (vol/vol) formalin in buffer A (0.1 M KCl/0.8 M glucose/5 mM $MgCl_2$/5 mM EGTA/10 mM Mops, pH 6.7) containing 0.05% Nonidet P-40 for 30 min. The slides were then immersed in buffer A containing 0.5% Nonidet P-40 for 1 h at room temperature. After washing with buffer A, the slides were incubated in buffer A containing rabbit anti-starfish egg myosin antiserum (8) or mouse monoclonal antibodies prepared against chicken brain α-tubulin (Amersham). After the unbound antibodies were removed by washing with buffer A, the slides were incubated with rhodamine-conjugated goat anti-rabbit IgG or fluorescein-conjugated goat anti-mouse IgG (Tago) dissolved in buffer A. Actin filaments were stained with 165 nM fluorescein-conjugated phalloidin or 33 nM rhodamine-conjugated phalloidin (Molecular Probes). Wheat germ agglutinin (WGA) receptors were visualized by staining with fluorescein-conjugated WGA (1 μg/ml; Vector Laboratories). To visualize DNA, the eggs were stained with 4′,6-diamidino-2-phenylindole (0.25 μg/ml). The chemicals were dissolved in buffer A. Isolation of egg cortices on protamine-coated glass surface was carried out as described (9). The sample was examined with a Nikon Optiphot microscope equipped with ordinary epifluorescence optics or confocal optics (MRC-600; Bio-Rad).

Isolation of the CR-Like Apparatus and Immunoblot Analysis. To isolate the CR-like apparatus, the CL-A-treated constricted eggs were suspended in 50 vol of buffer B [0.1 M KCl/5 mM $MgCl_2$/5 mM EGTA/0.5 mM dithiothreitol/leupeptin (10 μg/ml)/10 mM Mops, pH 6.7] containing 0.5% Triton X-100 for 10 min on ice. The egg suspension was passed through a nylon mesh (50 μm) and applied to a step gradient consisting of two layers of sucrose solution prepared in buffer B, 1 M at the top and 2 M at the bottom. After centrifugation at 1000 × *g* for 40 min at 4°C, the CR-like apparatuses were recovered at the interface between the two sucrose solutions and washed twice with buffer B by centrifugation at 1000 × *g* for 40 min at 4°C. To isolate the cortical layer from untreated unfertilized eggs, the eggs were homogenized in 50 vol of buffer B containing 0.5% Triton X-100 by using a hand-driven Teflon–glass homogenizer. The homogenate was centrifuged at 500 × *g* for 30 sec and the cortices were recovered in the pellet. They were washed twice with buffer B. The proteins of the isolated rings were resolved on a $NaDodSO_4$/polyacrylamide gel (10) and electrophoretically transferred to a nitrocellulose membrane (0.45-μm pore size;

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Abbreviations: CL-A, calyculin A; CR, contractile ring; MLC, myosin light chain; OA, okadaic acid; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; WGA, wheat germ agglutinin.

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Schleicher & Schüll). The membrane strips were incubated with the anti-myosin antiserum (8) in PBS (0.9% NaCl/10 mM sodium phosphate, pH 7.5) containing 0.05% Tween 20. The antigens were then visualized using the Vectastain ABC kit (Vector Laboratories).

Assay of Actin Polymerization. (*i*) CL-A (20 μM) was added to a rabbit skeletal muscle globular-actin solution (0.44 mg/ml) containing 20% (wt/wt) pyrene-labeled globular actin (11). KCl was added to 0.1 M to induce polymerization and the increase in the pyrene fluorescence was monitored. (*ii*) The egg cytoplasmic extract containing unpolymerized actin was prepared (12) from unfertilized eggs of *A. crassispina* in homogenizing buffer at pH 6.7 or 7.3, was incubated at 20°C for 90 min with or without 20 μM CL-A, and then centrifuged in an Airfuge (Beckman) at 30 psi (1 psi = 6.9 kPa) for 30 min. Both pellet and supernatant fractions were analyzed by $NaDodSO_4$/PAGE to detect actin bands.

Others. Phosphorylation of exogenously added H1 histone (type III-S; Sigma) in egg extracts was estimated as described (13). Protein synthesis was investigated by [^{35}S]methionine incorporation into a trichloroacetic acid-insoluble fraction of the eggs followed by $NaDodSO_4$/PAGE (14).

RESULTS

Cleavage-Like Morphological Change. When unfertilized *H. pulcherrimus* eggs were exposed to 20 μM CL-A dissolved in sea water, activation did not seem to occur: fertilization membrane formation was not observed and cortical granules examined by thin-section electron microscopy were found to remain (data not shown). However, in spite of the apparent absence of the activation process the eggs did undergo a series of morphological changes similar to those observed during the normal cleavage of fertilized eggs (Fig. 1). After the addition of CL-A to the egg suspension in ordinary sea water at 20°C, the eggs became slightly elongated at 50 min, began to constrict near the equatorial plane at 60 min, and cleaved into two fragments at 90 min. The "cleavage" usually took place in a plane close to the equator. In typical experiments, the ratio of diameters of the two fragments was between 1:1 and 2:1 in >95% of the eggs. A small number of the eggs underwent extremely unequal cleavage as in normal fertilized eggs. Similar results were obtained with eggs of *A. crassispina, Diadema savignyi, Diadema setosum, Echinostrephus aciculatus, P. depressus, Scaphechinus mirabilis, Stromopneustes variolaris, Temnopleurus hardwicki, Toxopneustes pileolus*. In contrast, most (>90%) eggs of *C. japonicus, Echinometra mathaei, Strongylocentrotus nudus*, and *Tripneustes gratilla* underwent extremely unequal cleavage and the rest (<10%) underwent almost equal cleavage. The fragments were connected by a transparent bridge and did not separate apart from each other. No further change was observed for 4 days. The time course of this process was dependent on temperature: it was completed within 40 min at 22°C, but it took 120 min at 18°C. The induced cleavage was irreversible. A CL-A pulse treatment for >5 min at 20°C could induce the cleavage. However, no morphological change was induced when the eggs were treated with 20 μM OA.

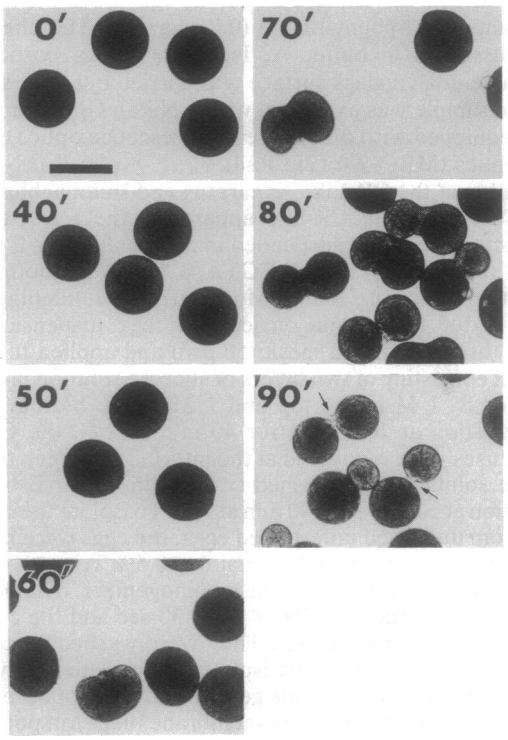

FIG. 1. Time course of morphological changes induced by CL-A. CL-A was added to a suspension of unfertilized *H. pulcherrimus* eggs in seawater at 20°C to a final concentration of 20 μM. Numbers indicate times after the addition of CL-A in minutes. Arrows in 90-min panel indicate transparent bridges. (Bar = 100 μm.)

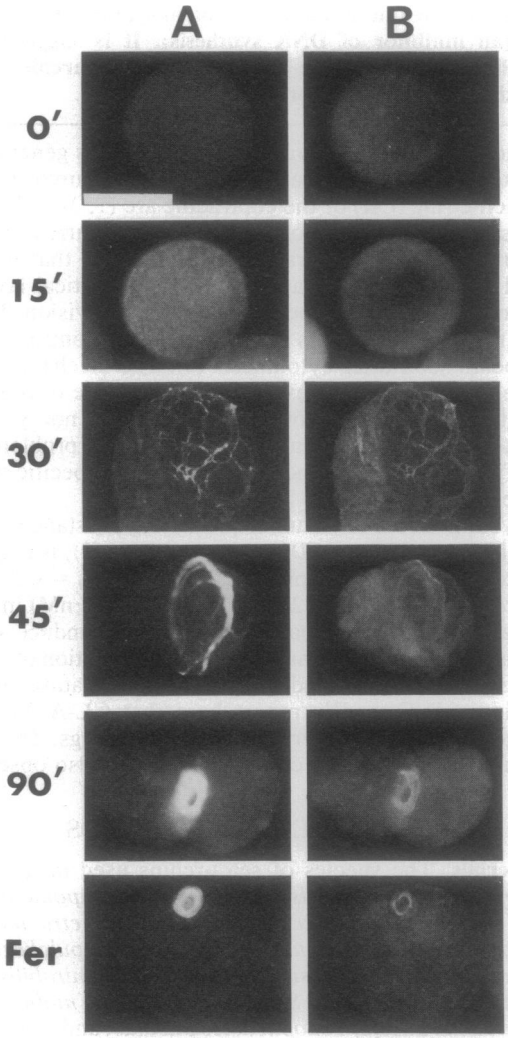

FIG. 2. Simultaneous localization of filamentous actin (*A*) and myosin (*B*) in the CL-A-treated unfertilized *H. pulcherrimus* eggs. Numbers indicate times in minutes after the addition of 20 μM CL-A at 20°C. Row Fer shows the CL-A-treated fertilized eggs 90 min after the treatment. (Bar = 100 μm.)

Microfilamentous Arrangement. The cleavage was completely inhibited by cytochalasin B (0.1–0.2 μM for *H. pulcherrimus* and *C. japonicus* and 0.6 μM for *P. depressus*), indicating that actin filaments were involved in this process. Thus, the process was investigated by fluorescence microscopy using fluorescently labeled phalloidin (Fig. 2*A*). Actin filament bundles appeared in the cortical layer 15 min after the addition of CL-A. These bundles then joined together into larger bundles (30 min). The arrangement of the larger bundles varied from egg to egg (compare Fig. 2*A* to Fig. 4*B*). The bundles fused into a cortical ring that surrounded the egg (45 min) and eventually constricted into a thicker smaller ring (90 min). This process was also confirmed by observing the actin filaments in egg cortices isolated on protamine-coated glass surface (data not shown). Myosin was colocalized with the actin filaments throughout the process (Fig. 2*B*). This CR-like apparatus was isolated and the concentration of both actin and myosin in the apparatus was confirmed by $NaDodSO_4$/PAGE (Fig. 3).

It has been reported (15) that lectin receptors move into the cleavage furrow of cultured mammalian cells. Accumulation of Con A receptors or WGA receptors in the furrow region of dividing sea urchin eggs has also been reported (16, 17). In the CL-A-treated eggs, the WGA receptors were colocalized with the actin filament bundles (Fig. 4 *A* and *B*).

It is plausible that CL-A inhibited the protein phosphatase activities and thereby elevated level of protein phosphorylation in the egg. The simultaneous addition with CL-A and 4 μM staurosporine (Kyowa Medex, Tokyo), an inhibitor of various protein kinases (18, 19), or 200 μM quercetin, an inhibitor of histone H1 kinase (20), protein kinase C (21), and cAMP-independent protein kinases (22), delayed the time of the cleavage; in both cases it took 210 min at 20°C. On the other hand, neither 0.5 mM ML-9, an inhibitor of myosin light chain (MLC) kinase (23), nor aphidicolin (10 μg/ml), an inhibitor of DNA synthesis (24), affected the cleavage.

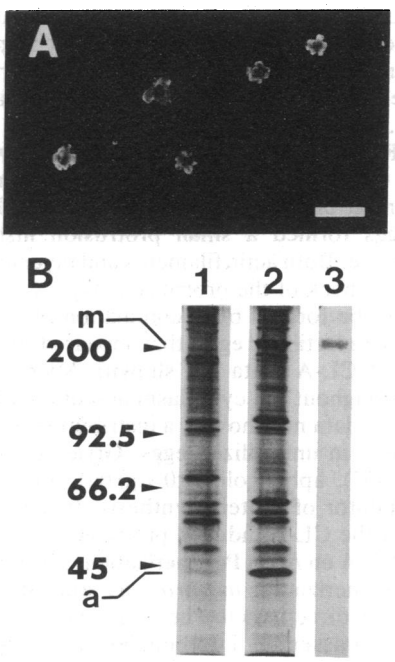

FIG. 3. Immunodetection of myosin in the isolated CR-like apparatuses of *H. pulcherrimus*. (*A*) Isolated CR-like apparatus stained with rhodamine-phalloidin. (Bar = 50 μm.) (*B*) Lanes: 1, silver staining of proteins in the cortices isolated from untreated eggs; 2, silver staining of proteins in the isolated CR-like apparatuses; 3, immunodetection of myosin. Numbers at the left indicate molecular mass in kDa. a, Actin; m, myosin.

Microtubular Structures. Microtubules were present up to 30 min after the CL-A treatment (Fig. 4*C*). As observed by confocal microscopy (Fig. 4*D*), the microtubules were scattered throughout the cytoplasm and did not form radiating

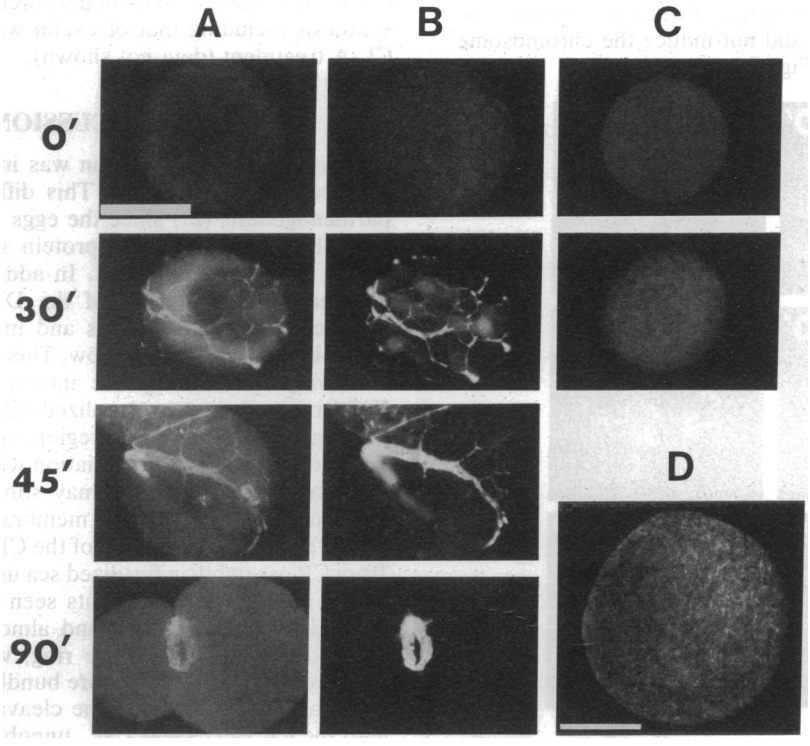

FIG. 4. Localization of WGA receptors and microtubules in CL-A-treated unfertilized *H. pulcherrimus* eggs. Numbers to the left indicate times in minutes after the addition of 20 μM CL-A at 20°C. (*A*) WGA receptors stained with fluorescein-WGA. (Bar = 100 μm.) (*B*) Filamentous actin stained with rhodamine-phalloidin in the same eggs as in *A*. (*C*) Immunofluorescence of microtubules in the CL-A-treated eggs. (*D*) Confocal immunofluorescence image (optical section) of microtubules in the CL-A-treated egg (50 min). (Bar = 50 μm.)

structures such as asters in the fertilized eggs. Furthermore, the CL-A-induced cleavage was not inhibited by griseofulvin, a microtubule inhibitor, even at high concentrations (500 μM), whereas normal cleavage was inhibited at <10 μM griseofulvin.

Effect on Fertilized Eggs. When fertilized eggs were treated with 20 μM CL-A at the time of insemination, they failed to undergo normal cleavage. As reported (25), CL-A-treated fertilized eggs formed a small protrusion instead of the normal cleavage. Both actin filaments and myosin were found in a ring at the neck of the protrusion (Fig. 2, row Fer). The ring seems to be formed by accumulation of large filament bundles, as in unfertilized eggs, that appeared 20–30 min after application of CL-A (data not shown). Microtubules were scattered throughout the cytoplasm and did not form radiating structures (data not shown), a distribution pattern similar to that formed in unfertilized eggs. Griseofulvin (500 μM), ML-9 (250 μM), aphidicolin (10 μg/ml), and emetine (100 μM), an inhibitor of protein synthesis, failed to inhibit the formation of the CL-A-induced protrusion.

Effect of CL-A on Actin Polymerization. The effect of CL-A on actin polymerization *in vitro* was studied to determine whether CL-A directly acted on actin and induced its polymerization and assembly into bundles in the egg. CL-A had absolutely no effect on the rate of polymerization of rabbit skeletal muscle actin. Furthermore, CL-A did not induce formation of any sedimentable actin (filamentous actin) in an egg cytoplasmic extract.

Chromosome Condensation. During the treatment of unfertilized eggs with CL-A, the condensation of chromosomes was observed (Fig. 5). At 20°C, the condensation was completed within 90 min. Neither segregation nor decondensation of the chromosomes occurred after the CL-A-induced condensation. The CL-A-induced chromosome condensation progressed more slowly in the presence of staurosporine or quercetin (data not shown). Treatment with 200 μM emetine, 500 μM griseofulvin, 500 μM ML-9, or aphidicolin (10 μg/ml) did not affect the progress of the CL-A-induced chromosome condensation. OA (20 μM) did not induce the chromosome condensation in the eggs (Fig. 5 *Lower Right*).

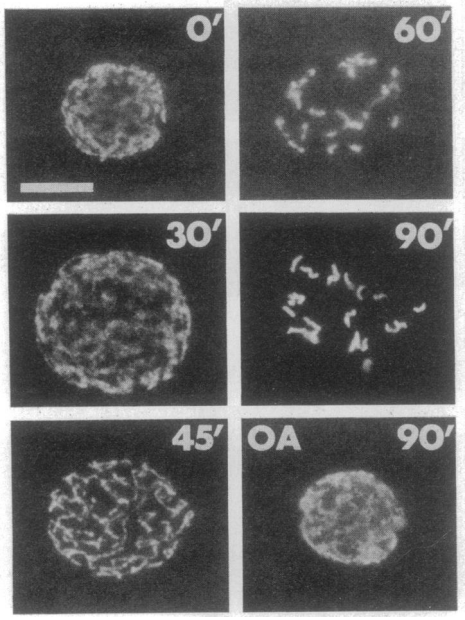

FIG. 5. CL-A-induced chromosome condensation in unfertilized *H. pulcherrimus* eggs stained with 4′,6-diamidino-2-phenylindole. Numbers indicate times in minutes after the addition of 20 μM CL-A at 20°C. The egg treated with 20 μM OA for 90 min is indicated OA. (Bar = 10 μm.)

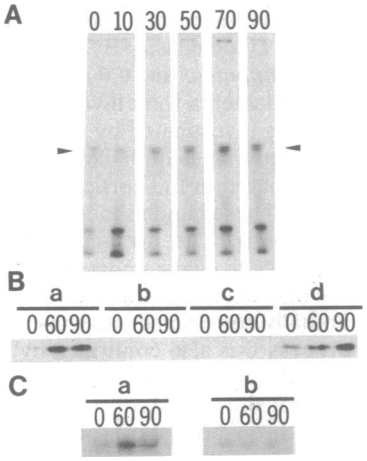

FIG. 6. Phosphorylation of histone H1 analyzed by $NaDodSO_4$/PAGE and fluorography. (*A*) CL-A (20 μM)-treated unfertilized *H. pulcherrimus* eggs at 20°C. (*B*) CL-A (20 μM)-treated unfertilized *Scaphechinus mirabilis* eggs at 21°C, without (lanes a) or with 4 μM staurosporine (lanes b), 200 μM quercetin (lanes c), or 200 μM emetine (lanes d). (*C*) Unfertilized *P. depressus* eggs treated with 20 μM CL-A (lanes a) or 20 μM OA (lanes b). Numbers indicate times in minutes after the addition of CL-A. Arrowheads indicate histone H1.

Histone H1 Kinase Activity and Protein Synthesis. Chromosome condensation is triggered by the phosphorylation of histone H1 (26). Thus, the activity of histone H1 kinase in extracts of the CL-A-treated eggs was measured. Exogenously added histone H1 was found to be phosphorylated in the extracts (Fig. 6). The time course of the phosphorylation was parallel to the chromosome condensation induced in live eggs. Addition of staurosporine or quercetin, but not emetine, to the eggs simultaneously with CL-A inhibited the phosphorylation of histone H1. OA did not activate the histone H1 kinase activity in the unfertilized eggs. No protein synthesis including that of cyclin was observed during the CL-A treatment (data not shown).

DISCUSSION

A cleavage-like phenomenon was induced by CL-A in unfertilized sea urchin eggs. This differed from well-known parthenogenesis (27) since the eggs did not develop further after the cleavage and no protein synthesis was observed during the CL-A treatment. In addition, the cleavage was induced in the presence of the DNA synthesis inhibitor aphidicolin. Actin filaments and myosin gathered concurrently into the cleavage furrow. Therefore, it is likely that the furrowing occurred by the interaction of these proteins. WGA receptors were colocalized with the actin filaments and condensed into the furrow region, suggesting that the WGA receptors are in close association with the actin filaments in the sea urchin egg. They may somehow anchor the actin filaments inside the plasma membrane.

The process of formation of the CR-like apparatus differed from CR assembly in fertilized sea urchin eggs. The bundling of the cortical actin filaments seen 30 min after incubation with CL-A was irregular and almost all of these bundles seemed to join to form the ring, whereas in the ordinary mitosis the actin filaments are bundled all over the cortex at anaphase and those near the cleavage plane are organized into the CR (ref. 9 and I.M., unpublished data). It has been proposed that two asters of the mitotic apparatus are required to establish the cleavage furrow in the normal cleavage (28, 29). Some unknown component(s) that stimulates the furrow formation is thought to move along astral microtubules to the

equatorial cortex (3). However, no aster-like structure was found in the CL-A-treated eggs. Furthermore, the cleavage induced by CL-A was not inhibited by the anti-microtubule drug griseofulvin. These results indicate that the CL-A-induced CR formation does not require the presence of microtubular structures. It is not clear how the cleavage plane is determined in the CL-A-induced cleavage. There is one more important feature of the CR-like apparatus: it did not disappear after the cleavage. This property differs from that of the normal cleavage furrow where the CR reduces its volume during constriction (4). It seems that the mechanism by which the CR disintegrates during constriction is not switched on in the CL-A-induced cleavage.

CL-A is known to strongly inhibit phosphatase activities of PP1 and PP2A. This inhibition would be expected to lead to increased levels of protein phosphorylation, thereby inducing actin filament assembly and formation of the CR-like apparatus. This assumption is supported by the fact that the cleavage was delayed by protein kinase inhibitors such as quercetin and staurosporine, which also inhibit the normal mitosis (30). CL-A-induced phosphorylation of various proteins has in fact been demonstrated in 3T3 fibroblasts (31). The phosphorylation of MLC is thought to be necessary for normal cleavage, since MLC kinase inhibitors (ML-7 and ML-9) inhibit cytokinesis (30). In contrast, however, CL-A-induced cleavage was not inhibited by ML-9. In chicken gizzard smooth muscle (32) that had been induced to contract by exposure to CL-A, dephosphorylation of MLC was inhibited, resulting in an increased level of its phosphorylation. This phosphorylation was shown to be different from the phosphorylation by MLC kinase since it did not require calcium or calmodulin. A similar MLC phosphorylation would be expected to occur in the CL-A-treated eggs. We hypothesize that the phosphorylation of MLC and/or some actin-modulating proteins may lead to actin polymerization, filament bundling, and the formation of the CR-like apparatus. The present result also suggests that the cleavage stimulus transduced from the mitotic apparatus to the cortex in the ordinary cytokinesis may be related to protein phosphorylation. We found that the isoelectric points of some proteins in newt eggs became more acidic upon entering the cleavage furrow (33), a change possibly due to phosphorylation of these proteins.

OA is known to inhibit PP2A activity at concentrations similar to those effective for CL-A (IC_{50} = 0.5–1 nM), whereas it is much less effective against PP1 in rabbit skeletal muscle (IC_{50} = 60–500 nM) (6). Even after treatment with 20 μM OA, no morphological change was observed in the sea urchin eggs, suggesting that CL-A induced the CR-like apparatus formation by affecting PP1 alone. In this respect, it is interesting that microinjection of PP1 into rat fibroblasts led to disassembly of the actin filament network (34).

The activation of histone H1 phosphorylation was observed in the CL-A-treated sea urchin egg extracts. CL-A also induces the phosphorylation of histone H1 in starfish oocytes (7). These data explain the induction of chromosome condensation by CL-A. However, in contrast to the starfish oocytes, there is no pool of cyclin B in unfertilized sea urchin eggs (35, 36), and no protein synthesis was observed during the CL-A treatment in this study. Furthermore, the activation of histone H1 kinase activity observed in the presence of CL-A was not affected by emetine. On the other hand, OA neither activates the H1 kinase activity nor induces chromosome condensation in sea urchin eggs. These results suggest that CL-A enhanced the histone H1 kinase activity in a cyclin B-independent manner by blocking the PP1 activity. This is dissimilar to the results obtained using frog prophase oocyte extracts, where inhibition of PP2A, but not PP1, was necessary to activate the histone H1 kinase (37). The results of our study are similar, however, to the results obtained with starfish oocytes where the maturation-promoting factor (histone H1 kinase) was found to be stabilized by inhibition of PP1 (13).

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