Mass isolation of cleavage furrows from dividing sea urchin eggs

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Summary

To develop a mass isolation procedure for the cleavage furrow from synchronized sea urchin eggs, we compared the stability of the cleavage furrow with that of the rest of the cortex (polar-region cortex) and the inner cytoplasm under various conditions using the rhodamine–phalloidin staining method. As a result, to remove the polar-region cortex and leave the cleavage furrow intact, it became clear that the type and concentration of detergent, the pH and Ca concentration of the isolation solution and the temperature were of critical importance, and that 0.04–0.1 % Nonidet P-40, pH 7.0–7.5, low calcium ion concentration and room temperature were optimal conditions. To solubilize the inner cytoplasm to release intact cleavage furrows, two factors, osmotic pressure and sea urchin species, were found to be important: 0.16 M glucose (or sucrose) was optimal, and we found *Clypeaster japonicus* to be the most appropriate. A shearing force, by gentle pipetting, was also required for furrow isolation. Taking these results into consideration, we have succeeded in developing a mass isolation procedure for cleavage furrow from *C. japonicus*. A total of 20–50 μg of protein of isolated cleavage furrow was recovered from 1 ml of packed dividing eggs. The structural integrity of the isolated cleavage furrow was well maintained and it was characterized by remnants of plasma membranes, actin filament meshwork including a contractile ring, and cytoplasmic vacuoles. Although the isolated furrow contained myosin II molecules, it showed no capability of *in vitro* reactivation.

Key words: cleavage furrow, cortex, contractile ring, rhodamine–phalloidin, sea urchin egg.

Introduction

The mechanism of cytokinesis has attracted increasing interest among cell biologists. It is now widely accepted that just beneath the plasma membrane at the cleavage furrow a band of aligned microfilaments called the contractile ring or arc is formed prior to and during cytokinesis (for review see Mabuchi, 1986), and that this band exerts a mechanical force for cleavage through direct interaction between myosin II and actin filaments (Mabuchi and Okuno, 1977; De Lozanne and Spudich, 1987; Knecht and Loomis, 1987). This contractile ring or arc was reported to occur in various types of animal cells including protozoa. Therefore, for a better understanding of the molecular mechanism of cytokinesis the following questions must be answered: what kinds of cytoskeletal proteins constitute the contractile ring? How are actin filaments in the contractile ring associated with the plasma membrane at the cleavage furrow? What is the signal for triggering the formation of the ring and for determining the position of the ring? How is the ring destroyed in accordance with the stage of cleavage?

To answer these questions, intensive studies have been made. Immunofluorescence microscopy has revealed that some actin-binding proteins such as myosin II (Fujiwara and Pollard, 1976; Yumura and Fukui, 1985; Schroeder and Otto, 1988), α-actinin (Fujiwara *et al.* 1979; Mabuchi *et al.* 1985; Mabuchi *et al.* 1988) and filamin (Nunnally *et al.* 1980) are concentrated at the contractile ring. Radixin, a barbed-end-capping actin-modulating protein, was recently identified as a candidate for binding the barbed end of actin filaments to the plasma membrane at the furrow (Sato *et al.* 1991). Recent microinjection studies made it possible to analyze the dynamic behavior of these cytoskeletal proteins, including actin molecules, during formation and destruction of the contractile ring (Sanger *et al.* 1987; Wang, 1987; Sanger *et al.* 1989; Cao and Wang, 1990). Upon stimulation of contractile ring formation, it was shown that the astral microtubules might play an important role, and recently two distinct models for astral stimulation of cytokinesis were proposed (Devore *et al.* 1989; Harris and Gewalt, 1989). In spite of these studies, however, our knowledge of the molecular mechanism of cytokinesis is still fragmentary.

To complete the picture of cytokinesis at the molecular level, isolation and characterization of cleavage furrows from dividing cells would be an ideal approach: in other actin-containing cytoskeletal systems such as brush borders of intestinal epithelial cells (Tilney and Mooseker, 1971), the circumferential actin filament bundles of

epithelial cells (Owaribe and Masuda, 1982), and cell-to-cell adherens junctions of liver cells (Tsukita and Tsukita, 1988), the development of isolation procedures gave us a large amount of information on the molecular organization and functions of these structures. When we try to isolate cleavage furrows, we face two technical difficulties, since the cleavage furrow is a transitory structure: (1) the structure may be too labile for isolation, and (2) it may be difficult to isolate furrows in large amounts. So far the first difficulty has been overcome only in newt eggs: cleavage furrows were isolated surgically from dividing newt eggs with a fine glass needle, and their structural integrity was maintained (Perry *et al.* 1971; Mabuchi *et al.* 1988). However, as expected, it is difficult to isolate a large number of cleavage furrows from newt eggs by such a surgical method. To overcome the second difficulty, sea urchin eggs appear to be an excellent source of cleavage furrows, because they are obtained in large amounts and they divide synchronously. Therefore, if the cleavage furrow of sea urchin eggs is not too labile, mass isolation of cleavage furrows would be possible from sea urchin eggs. However, to date it was widely believed that the contractile ring lining the cleavage furrow was too fragile to be isolated in sea urchin eggs (Schroeder, 1981).

A recent improvement in the detection of actin filaments at the light-microscopic level, the rhodamine–phalloidin (Rh–ph) staining method (Faulstich *et al.* 1983), has made it possible to detect the cleavage furrow in sea urchin eggs easily and quickly. In this study, taking advantage of this staining method, we first showed that under appropriate conditions the cleavage furrow of sea urchin eggs is stable enough for isolation. Secondly, we determined the various kinds of factors that would affect the stability of cleavage furrows. Finally, taking these factors into consideration, a new mass isolation procedure for the cleavage furrow was developed using synchronized sea urchin eggs. We believe that this procedure will lead to a new way of studying the molecular mechanism of cytokinesis in animal cells.

Materials and methods

Cell culture

Four types of sea urchin, *Hemicentrotus pulcherrimus*, *Anthocidaris crassispina*, *Clypeaster japonicus* and *Strongylocentrotus nudus* were used in this study. Eggs and sperm were obtained by intracoelomic injection of 0.1 M acetylcholine. Eggs were washed twice with ordinary sea water. In *C. japonicus*, eggs were further washed with Ca-free sea water until the eggs were dejellied. After insemination in Ca-free sea water, fertilization membranes were removed using 74 μm nylon mesh (Yonemura and Kinoshita, 1986). In other other species, eggs were inseminated in ordinary sea water. One minute after insemination, 9 volumes of 1 M urea was added and fertilization membranes were removed with the same nylon mesh. After being washed three times with Ca-free sea water, the fertilized eggs were cultured in Ca-free sea water in a watch glass.

Isolation of egg cortices on coverslips

Cortices of dividing eggs were isolated on protamine sulfate-coated coverslips according to the method described previously (Yonemura and Kinoshita, 1986; Yonemura and Mabuchi, 1987).

Cleavage furrow isolation medium

The standard buffer solution for cleavage furrow isolation used in this study consisted of 0.1 M KCl, 2 mM $MgCl_2$, 5 mM EGTA, 0.5 mM dithiothreitol, 5 μg ml^{-1} leupeptin and 10 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) buffer (pH 7.3). Nonidet P-40 (NP-40), Triton X-100, saponin and sodium deoxycholate were used as detergents. When pH was adjusted to 6.0, 6.5 and 7.5–8.0, 10 mM Mes, Pipes and Hepes were, respectively, used instead of 10 mM Mops. Unless otherwise mentioned, to check the effects of various kinds of medium on the morphology of the cleavage furrow, 80 μl of a dense suspension of dividing eggs from the bottom of a watch glass was added and mixed with 2 ml of the medium in a 10 ml test tube at room temperature. The eggs were allowed to settle for 20 min, and a small sample of the eggs was removed for light-microscopic examination using rhodamine–phalloidin staining.

Staining with rhodamine–phalloidin (Rh–ph)

Egg cortices isolated on coverslips were fixed with 1% formaldehyde and stained with 0.3 μM Rh–ph (Molecular Probes, Inc., Junction City, OR), which binds to filamentous actin (Faulstich *et al.* 1983), as described previously (Yonemura and Kinoshita, 1986; Yonemura and Mabuchi, 1987). To judge whether or not cleavage furrows were isolated intact, 10 μl of the sample was immediately stained for 20 min without fixation by adding an equal volume of 0.3 μM Rh–ph to the standard buffer solution. For observation of the process of cleavage furrow isolation, *C. japonicus* eggs were fixed with 2% formaldehyde dissolved in isolation medium containing 0.1% NP-40 and 0.16 M sucrose for 20 min at room temperature. They were washed with phosphate-buffered saline (PBS: 150 mM NaCl, 10 mM phosphate buffer, pH 7.2) three times and stained with 0.3 μM Rh–ph in PBS for 20 min. Specimens were observed under a Nikon Optiphot microscope equipped with epifluorescence and phase-contrast optics (XF-EFD-ph, Nikon, Tokyo, Japan) with CF Fluor DL 10× (NA 0.5) or CF Fluor DL 40× (NA 0.85). Photographs were recorded on Kodak Tri-X pan film at an exposure of 0.25–3.0 s, and the film was developed at 1600 ASA.

Electron microscopy

The fraction rich in cleavage furrows obtained after sucrose gradient centrifugatin was fixed with 2.5% glutaraldehyde, 0.1% tannic acid, 2 mM $MgCl_2$, 5 mM EGTA, 0.1 M Pipes buffer (pH 6.8) for 2 h at room temperature followed by post-fixation with 0.1 M cacodylate buffer (pH 7.4) containing 1% OsO_4 for 1 h at 4 °C. The samples were then dehydrated with ethanol and embedded in Epon 812. Thin sections were cut with a diamond knife, doubly stained with lead citrate and uranyl acetate, and then examined in a JEOL electron microscope (JEM 1200 EX).

Electrophoresis

The fraction rich in isolated cleavage furrows from *C. japonicus* eggs was mixed with an equal volume of the standard buffer solution and then centrifuged at 12 000 ***g*** for 5 min at 4 °C to pellet the furrows. Cortices of *C. japonicus* eggs at late prophase were isolated as described previously (Mabuchi *et al.* 1980). Two-dimensional gel electrophoresis was carried out essentially according to O'Farrell (1975), with modifications. The first-dimensional gel containing 2% Ampholine (pH 5–7:pH 3.5–10=4:1) was made in a hematocrit tube (cut at 4.5 cm long) at a height of 3.5 cm. Isoelectric focusing was carried out at 300 V for 3 h. Gel rods were then placed on the second-dimensional gel slabs (10% acrylamide; 0.8 mm thick, 7 cm wide and 5 cm in height). After sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Laemmli, 1970), the slab gels were stained with ammoniacal silver (Oakley *et al.* 1980). Sea urchin egg actin (Mabuchi and Spudich, 1980) was used as a standard protein.

Immunoblotting

The furrow fraction was electrophoresed in a 8% polyacrylamide slab gel in the presence of SDS and then electrophoretically transferred onto a nitrocellulose membrane (Katayama *et al.* 1984). Proteins on the membrane were stained with 0.1% Amido Black. The cross-reactivity of antibodies against starfish oocyte myosin II (Mabuchi and Okuno, 1977) with the transferred proteins was tested by the immunoblotting method using a Vectastain ABC kit (Vector Labs. Inc., Burlingame, CA).

Results

Stability of cleavage furrows in dividing sea urchin eggs

To date it was widely believed that the contractile ring in dividing sea urchin eggs was too fragile to be isolated with its structural integrity intact. However, as described below, when we observed the morphology of the contractile ring of sea urchin eggs in various kinds of preparations using the rhoadmine–phalloidin staining method, we obtained some evidence indicating that it might be stable under some appropriate conditions.

The first evidence was obtained when egg cortices of the sea urchin *H. pulcherrimus* were isolated on a substratum. Dividing eggs were stuck on protamine sulfate-coated coverslips and subjected to a jet stream of the standard buffer solution. In this type of preparation, more than half of the cortex of each egg was removed together with the inner cytoplasm, leaving some part of the cortex on a coverslip. As shown in Fig. 1A–D, in the cortex isolated on a glass, which included a cleavage furrow, the furrow region was occasionally observed to be associated with a long actin bundle that was intensively stained with Rh–ph. Judging from its morphology and orientation on the isolated cortex, it appears to be reasonable to regard this actin bundle as a contractile ring. Therefore, it may be safe to say that the cleavage furrow is more resistant to shearing forces than other parts of the cortex under the conditions used in this experiment.

The second evidence was obtained when the actin filament organization of dividing sea urchin eggs was analyzed after treatment with various kinds of detergents. As shown in Fig. 1E and F, when *H. pulcherrimus* eggs were treated with the standard buffer solution plus 0.05 % NP-40 and 0.8 M glucose followed by gentle pipetting, the polar-region cortex was peeled off and the contractile ring remained intact at the cleavage furrow (see Fig. 3A for intact cortices). Similar results were obtained with three other species.

These observations led us to conclude that under appropriate conditions the contractile ring of sea urchin eggs was fairly stable even in the absence of the inner cytoplasm (see Fig. 1A–D) and the polar-region cortex (see Fig. 1E,F), and persuaded us to try to develop a mass isolation procedure for cleavage furrows from sea urchin eggs. This isolation procedure should theoretically contain two steps, although these two steps may actually occur simultaneously (Fig. 2). The first step is to remove selectively the polar-region cortex, leaving the cleavage furrow intact on the inner cytoplasm. The second step is to solubilize the inner cytoplasm to release isolated cleavage furrows. Therefore, we first attempted to clarify precisely the appropriate conditions required for each step.

Conditions to remove selectively the polar-region cortex, leaving the cleavage furrow intact

As will be described below, when 0.8 M sucrose was included in the isolation solution, the inner cytoplasm was found to be fairly stable against shearing forces, indicating that the second step in Fig. 2 could be selectively inhibited. Therefore, at first, using isolation solution containing 0.8 M sucrose, we attempted to analyze precisely the conditions required specifically for the first step in Fig. 2.

Detergent and shearing force. H. pulcherrimus eggs were treated with the standard buffer solution containing various concentrations of NP-40 followed by Rh–ph staining. At higher concentrations (0.1–0.2 %) of NP-40, the actin filament-containing cytoskeleton both at the cleavage furrow and the polar-region appeared to be fairly stable (Fig. 3A), while NP-40 at lower concentrations (0.04–0.06 %) caused a partial peeling-off of the polar-region cortex leaving the cleavage furrow (or contractile ring) intact (Fig. 3B). This concentration-dependent effect of NP-40 on the polar-region cortex became much clearer after shearing treatment of the NP-40-treated cells by gentle pipetting. At lower concentrations the polar-region cortex was peeled off almost completely, leaving intact contractile rings on inner cytoplasm (Fig. 3D), but this did not occur at higher concentrations (Fig. 3C). Passing the NP-40-treated eggs through a 23-gauge needle was also effective. Use of a narrower needle and vigorous pipetting resulted in destruction of cleavage furrows. NP-40 at much lower concentrations (0.02 %) was not effective in peeling-off the polar-region cortex. Triton X-100 (0.02–1 %) was slightly less effective than NP-40. Neither saponin (0.001–0.1 %) nor sodium deoxycholate (0.01–2 %) was effective for the preservation of actin filament-containing structures. Similar results were obtained with two other species (*A. crassispina* and *S. nudus*). With *C. japonicus*, 0.1 % NP-40 was the most effective.

pH. The pH of the isolation medium was one of the important factors in the removal of the polar-region cortex while maintaining the structural integrity of the cleavage furrow. When dividing eggs were treated with an appropriate concentration of detergent followed by gentle pipetting, under acidic conditions (pH 6.0–6.5), not only the polar-region cortex but also the cleavage furrow was destroyed. On the other hand, under alkaline conditions (pH 8.0) the preservation of the contractile ring was good but the peeling-off of the polar-region cortex was not complete. Therefore, it was concluded that pH 7.0–7.5 was optimal for cleavage furrow isolation. We chose pH 7.3 for the following experiments because it was reported to be the physiological pH in fertilized sea urchin egg cytoplasm (Shen and Steinhardt, 1978).

Temperature. When we treated eggs at low temperatures (e.g. 10 °C, 4 °C or 0 °C), the furrows could not be separated effectively, suggesting that the egg cortex changes in its mechanical properties in a temperature-dependent manner. For example, when dividing *C. japonicus* eggs were treated at 0 °C with NP-40, followed by gentle pipetting, the polar-region cortex appeared to be too stabilized to be peeled off and in contrast the contractile ring disappeared. Thus, treatment of cells with detergent must be performed at room temperature (for *C. japonicus* and *A. crassispina*, 25–29 °C; for *H. pulcherrimus* and *S. nudus*, 16–20 °C). Once the polar-region cortex was peeled off, the low temperature did not affect the furrows.

Other factors. In accordance with the biochemical results already reported (Spudich and Spudich, 1979), EGTA (5 mM) was indispensable in the isolation medium, since actin filament-containing structures were not preserved in the presence of calcium ion. Changing the concentrations of KCl and $MgCl_2$ (50 mM–0.2 M and 1–10 mM, respectively) did not have any effect. Glycerol and dimethyl sulfoxide were effective in preserving the furrow with *S. nudus* only.

Conditions for solubilizing the cytoplasm to release intact cleavage furrows

Next, conditions required for the solubilization of the inner cytoplasm of dividing eggs were analyzed (the

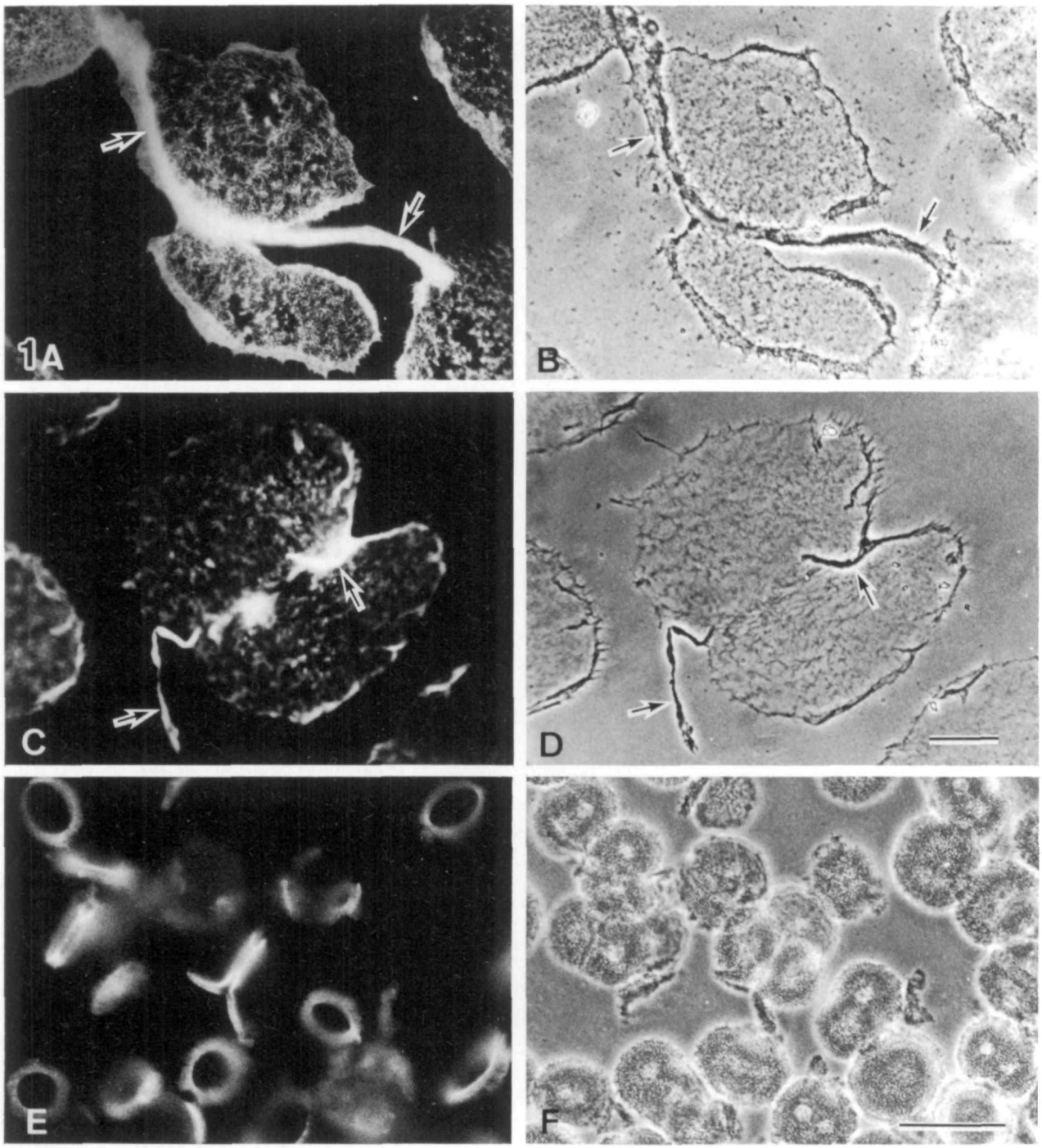

Fig. 1. Stability of the cleavage furrow under two different conditions. Fluorescence (A,C,E) and phase-contrast (B,D,F) micrographs of isolated cortices from dividing *H. pulcherrimus* eggs stained with Rh–ph. (A–D) A large part of the cleavage furrow (arrows) remained on the coverslip while more than half of the cortex was removed together with the inner cytoplasm. Bar, 20 μm. (E,F) Cleavage furrows remained on dividing *H. pulcherrimus* eggs stained with Rh–ph after treatment with isolation medium containing 0.05% NP-40 and 0.8 M glucose, followed by gentle pipetting. The polar-region cortex had peeled off (see Fig. 3A for intact cortices). Bar, 100 μm.

second step in Fig. 2). As a result, it became clear that a high shearing force, low temperature, sodium deoxycholate and low osmotic pressure of the isolation solution were effective for solubilizing the inner cytoplasm. However, the first three treatments did not permit preservation of the cleavage furrow.

Osmotic pressure. Fig. 4 shows the effect of the osmotic pressure on *C. japonicus* eggs. When the osmotic pressure of the isolation solution was isotonic to sea water (0.8 M glucose in the solution), the inner cytoplasm did not collapse against gentle pipetting (Fig. 4A,B). When the osmotic pressure was low (0.16 M glucose), the inner

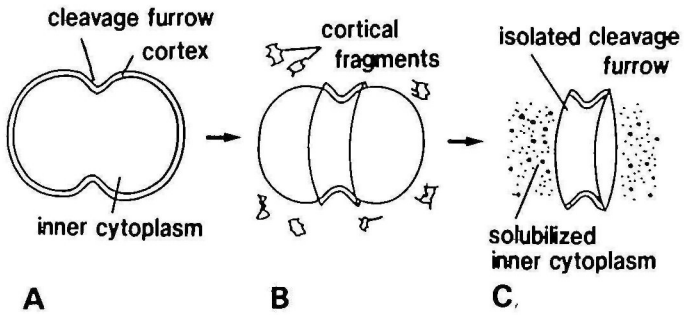

Fig. 2. Schematic drawing of the procedure for cleavage furrow isolation. (A) A dividing cell. (B) The first step. The polar-region cortex has peeled off from the cell. (C) The second step. The inner cytoplasm is solubilized, while the cleavage furrow is intact.

cytoplasm broke into pieces by low shearing forces (Fig. 4C,D). When the osmotic pressure was further lowered (0–0.08 M glucose), the intact cleavage furrows decreased in number. Sucrose had a similar effect on the breakdown of the inner cytoplasm. Glycine and fructose were not good for preserving the furrow.

Species of sea urchin. Treatment of dividing eggs with a solution containing several appropriate components as described above, followed by gentle pipetting, was found to be effective in isolating cleavage furrows. However, its efficiency depended on species. In *H. pulcherrimus*, for example, when we lowered the concentration of glucose from 0.8 M to 0.16 M, the recovered intact cleavage furrows decreased in number as the inner cytoplasm was solubilized. Therefore, we searched for suitable species and found that *C. japonicus* was the most appropriate for cleavage furrow isolation. The effects of chemical and physical factors on the isolation of cleavage furrows from this species are summarized in Table 1.

Mass isolation of cleavage furrows from C. japonicus *eggs*

Hand-centrifuged pellets (1–10 ml) of dividing *C. japonicus* eggs were mixed with 50 volumes of the standard buffer solution plus 0.1 % NP-40 and 0.16 M sucrose, and then allowed to settle in a watch glass for 20 min at room temperature (25–29 °C). The extracted eggs were collected at the bottom of a watch glass and most of the supernatants were removed. The sediments were gently sheared by pipetting, to form a dense suspension. Eggs that did not lyse were removed by passing the suspension through two layers of 49 μm nylon mesh. The suspension was then layered on a discontinuous sucrose gradient consisting of 1.5 M and 0.3 M sucrose dissolved in the

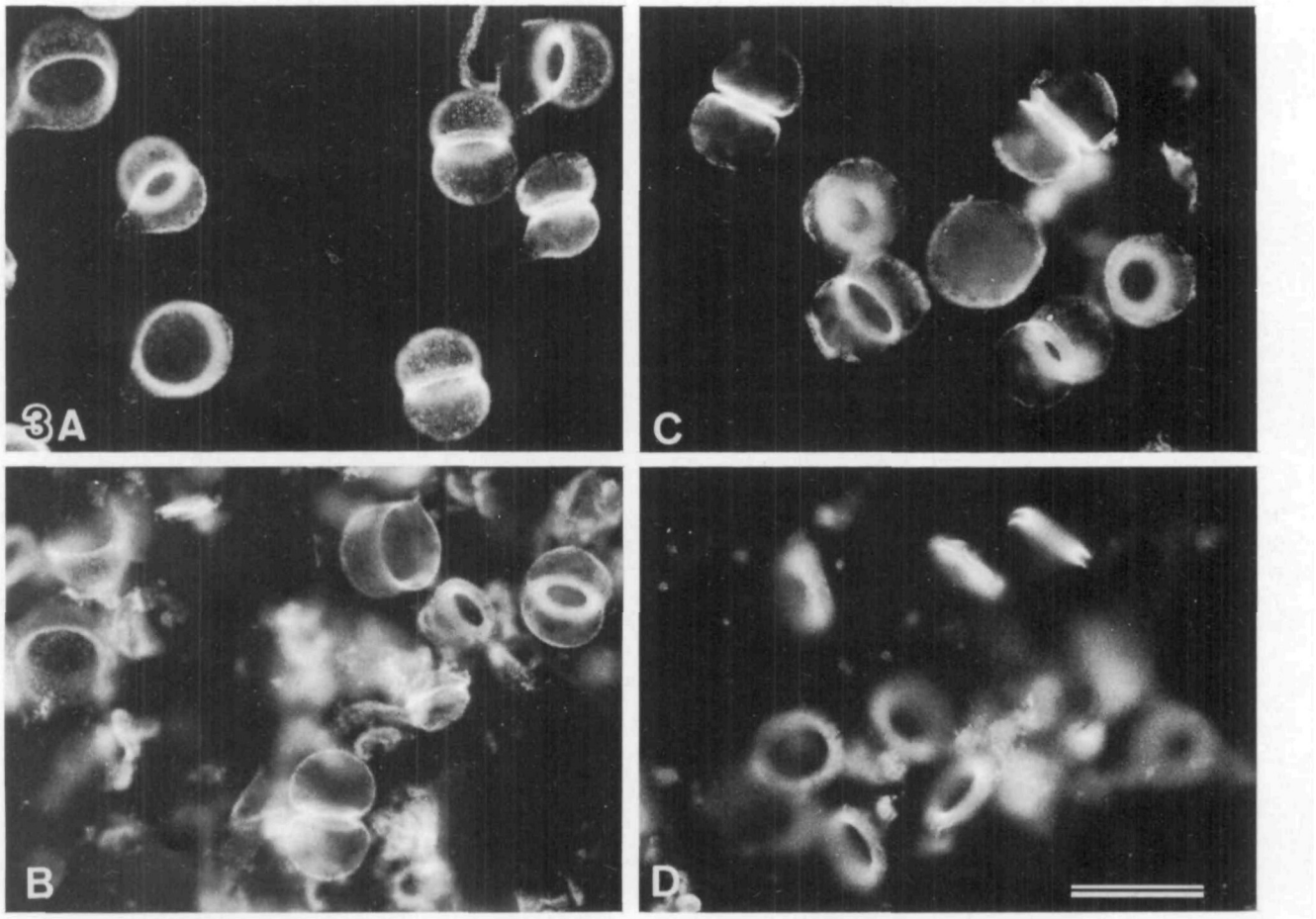

Fig. 3. Effects of the concentration of NP-40 and shearing force on cleavage furrow isolation from *H. pulcherrimus* eggs. (A,C) Dividing eggs treated with isolation medium containing 0.1 % NP-40 and 0.8 M glucose. (B,D) Dividing eggs treated with isolation medium containing 0.04 % NP-40 and 0.8 M glucose. (C,D) Eggs sheared by gentle pipetting after detergent treatment. All samples were stained with Rh–ph. The combination of 0.04 % NP-40 and shearing (D) was the most effective of the four treatments in peeling off the polar-region cortex, while maintaining the cleavage furrow intact. Bar, 100 μm.

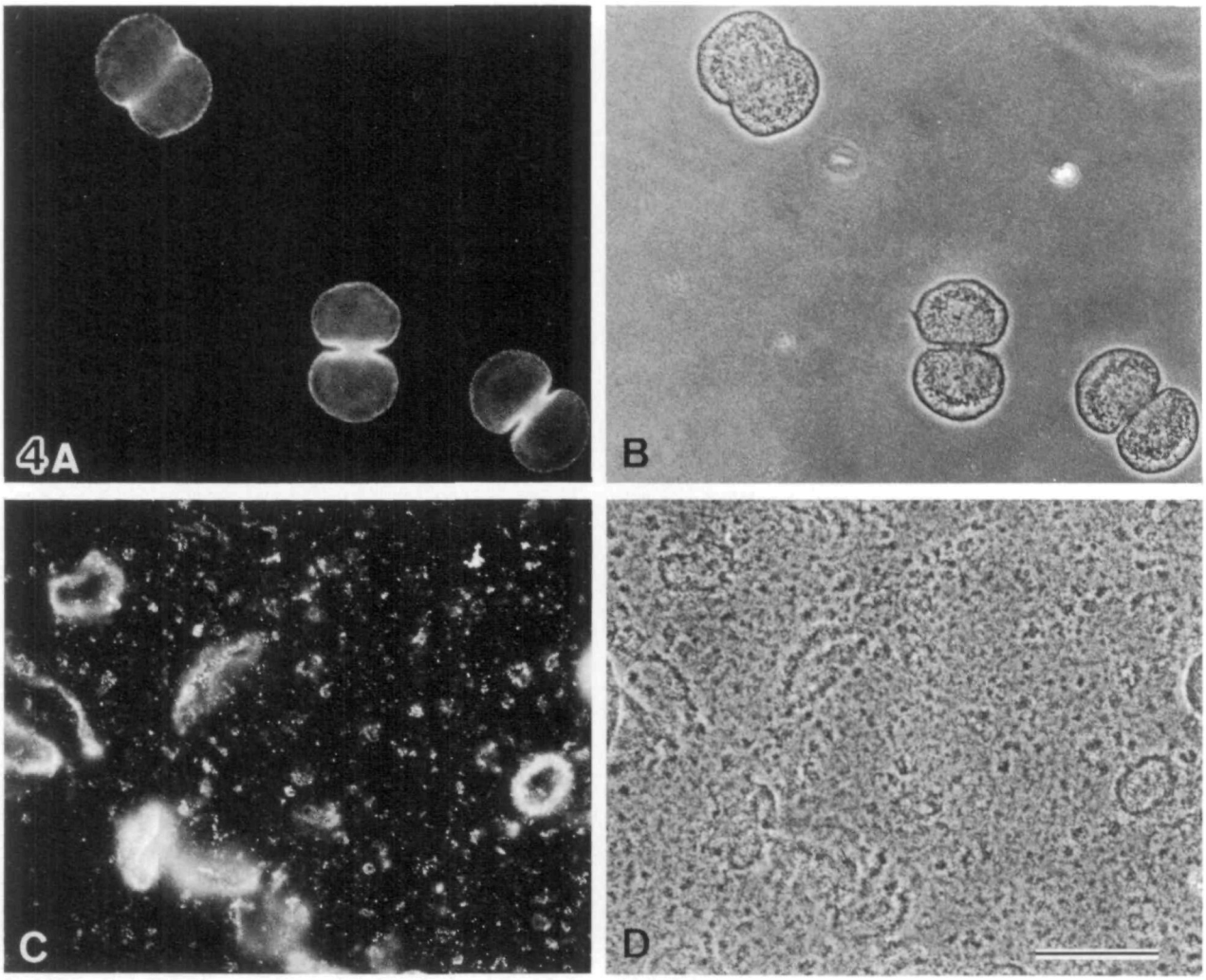

Fig. 4. Effect of the osmotic pressure of the isolation medium in solubilizing the inner cytoplasm of *C. japonicus* eggs. Fluorescence (A,C) and phase-contrast (B,D) micrographs. Dividing eggs treated with the isolation medium containing 0.1 % NP-40 and 0.8 M glucose (A,B), or 0.1 % NP-40 and 0.16 M glucose (C,D). The inner cytoplasm was effectively solubilized when the osmotic pressure was lowered (C,D). Bar, 100 μm.

isolation solution and centrifuged at 15 ***g*** for 30 min at 4 °C. The volumes of the tubes used for this centrifugation were 10–40 ml. A fraction rich in cleavage furrows was recovered at the 0.3 M/1.5 M interface. This procedure yielded an isolated furrow fraction containing 20–50 μg of protein from 1 ml of packed eggs.

Fig. 5 illustrates the process of separation of the cleavage furrow from the bulk of the cortex and the inner cytoplasm. Two minutes after the onset of the treatment, the polar-region cortex was peeled off and simultaneously most of the inner cytoplasm was deformed but not completely dispersed (Fig. 5C,D). When these lysed dividing eggs were gently sheared by pipetting, cleavage furrows and small fluorescent pieces (cortical fragments) were seen as actin filament-containing structures among enormous amounts of cytoplasmic material (Fig. 6A,B). After filtration through layers of nylon cloth followed by sucrose density gradient centrifugation, cleavage furrows were recovered at 0.3 M/1.5 M interface (Fig. 6C–F). Most of the small cytoplasmic material remained in the top layer, and small cortical fragments were recovered mainly in the 0.3 M sucrose layer under these centrifugation conditions. The isolated furrows seemed to maintain their *in situ* morphology. Only a few cytoplasmic particles and cortical fragments were detected as contaminants in the purified furrow fraction by phase-contrast microscopy. Prolonged centrifugation caused contamination of small cortical fragments in the furrow fraction. When the sample was centrifuged at gravities higher than 15 ***g*** (100–100 000 ***g***), cleavage furrows were tangled together or disrupted so that they were hardly distinguishable from clots of cortical fragments. Egg cortices at stages other than telophase were so stable that they were often found in the cleavage furrow fraction as contaminants. Therefore, highly synchronized eggs should be used as starting material.

Morphology, protein composition and reactivation of isolated cleavage furrows

The morphology of isolated furrows from *C. japonicus* was examined by fluorescence microscopy (Fig. 7). The furrow diameter was variable, probably according to the stage of constriction at which the furrows were isolated. There was no difference in fluorescence intensity along the furrow.

Table 1. *Effects of chemical or physical factors on the isolation of cleavage furrows from* C. japonicus *eggs*

Factors		Efficiency
Detergents: NP-40(%)	0.02	−
	0.05	+
	0.1	++
	0.2	+
Triton X-100(%)	0.05	+
pH	6.0	−
	6.5	+
	7.0–7.5	++
	8.0	+
Temperature	Room temperature (25–29 °C)	++
	0 °C	−
Ions	1 mM Ca^{2+}	−
	5 mM EGTA	++
	1–10 mM $MgCl_2$	++
	50–200 mM KCl	++
Osmotic pressure	0.8 M glucose	−
	0 16 M glucose, sucrose	++
	0.08–0 M glucose, sucrose	+
	Glycine, fructose	−
Gravity at density gradient centrifugation	100–100 000 *g*	−
	15 *g*	++

−,+ and ++: poor, moderate and high efficiency of cleavage furrow isolation, respectively.

Parallel arrays of actin filament bundles of the contractile ring could not be recognized under the present optical conditions. Furrows fairly large in diameter were often folded (Fig. 7D) or broken into pieces during isolation, suggesting that they were more fragile than those in later stages of constriction. From an egg dividing into four cells at the first cleavage because of polyspermy, two cleavage furrows combined at right angles were isolated (Fig. 7H).

The furrows were further examined by electron microscopy (Fig. 8A). The isolated furrow was characterized by remnants of the plasma membrane, a layer of actin filament meshwork and cytoplasmic vacuoles. At the bottom of the furrow, a layer of densely packed actin filaments was seen (Fig. 8B). The thickness and the width of this layer are about 0.2 μm and 4 μm, respectively. In some cases, as shown in Fig. 8B, this layer was seen to be divided into several tight bundles, indicating that the contractile ring might be composed of several bundles. The center-to-center spacing of the filaments in these bundles was 8–14 nm.

Next, the two-dimensional gel pattern of isolated furrows from *C. japonicus* was compared with that of egg cortices isolated before cleavage (Fig. 9A,B). Interestingly, the isolated cleavage furrow appeared to differ in its molecular organization from the cortices isolated before cleavage: there were several proteins that were unique to the furrows or to the cortices before cleavage. Myosin II was detected in the isolated cleavage furrows by immunoblotting analysis using antibodies against starfish oocyte myosin II (Fig. 9C,D), indicating that myosin II molecules were not lost from the furrows during the procedure.

The question has naturally arisen of whether these isolated cleavage furrows can be reactivated *in vitro*. We

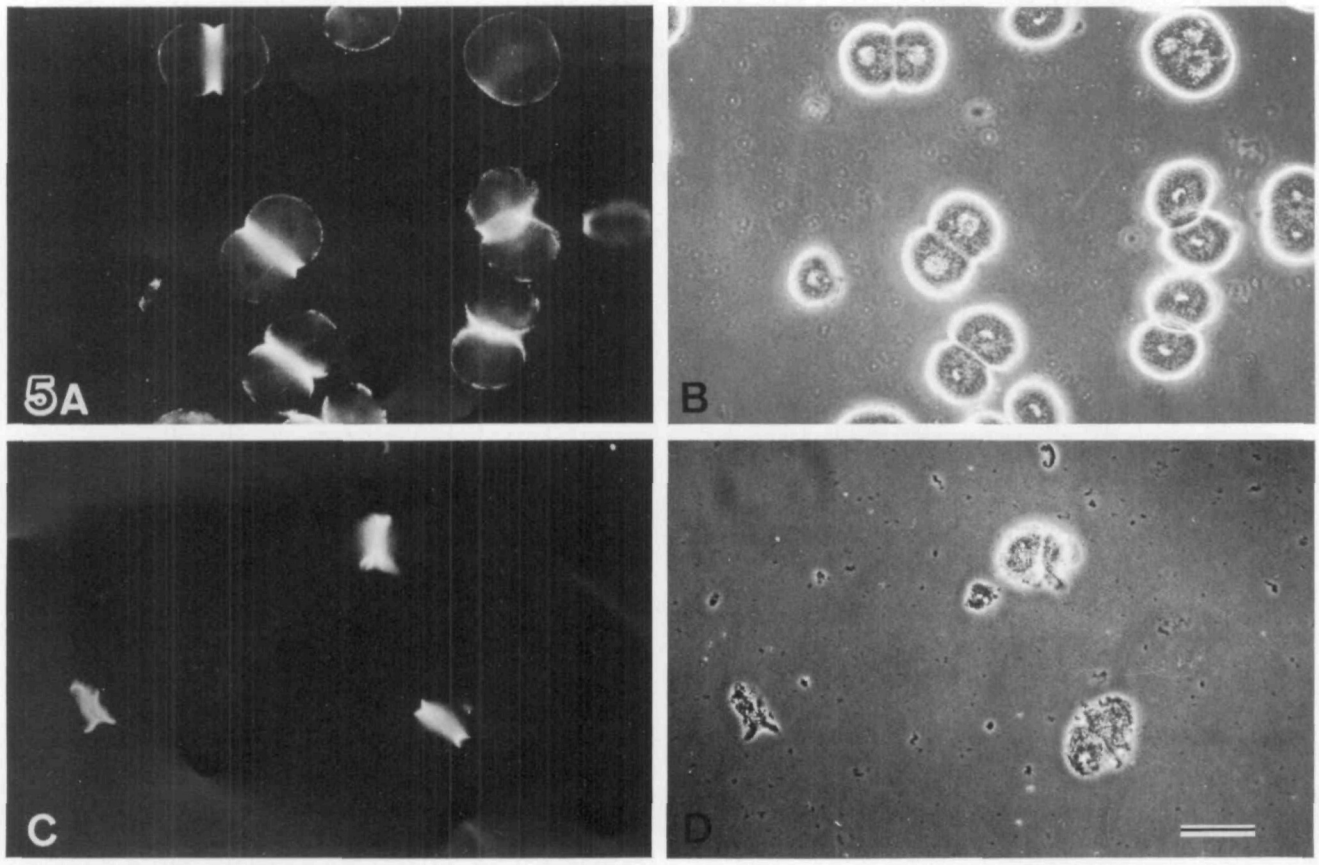

Fig. 5. *C. japonicus* eggs in isolation medium stained with Rh–ph, showing the process of the isolation. Fluorescence micrographs (A,C) and phase-contrast micrographs of the same field (B,D), respectively. (A,B) Dividing eggs were treated with the isolation medium plus formaldehyde. Cleavage furrows are stained intensely. (C,D) Dividing eggs were treated with the isolation medium for 2 min and then fixed. The polar-region cortex was peeled off and most of the inner cytoplasm began to disperse. Bar, 200 μm.

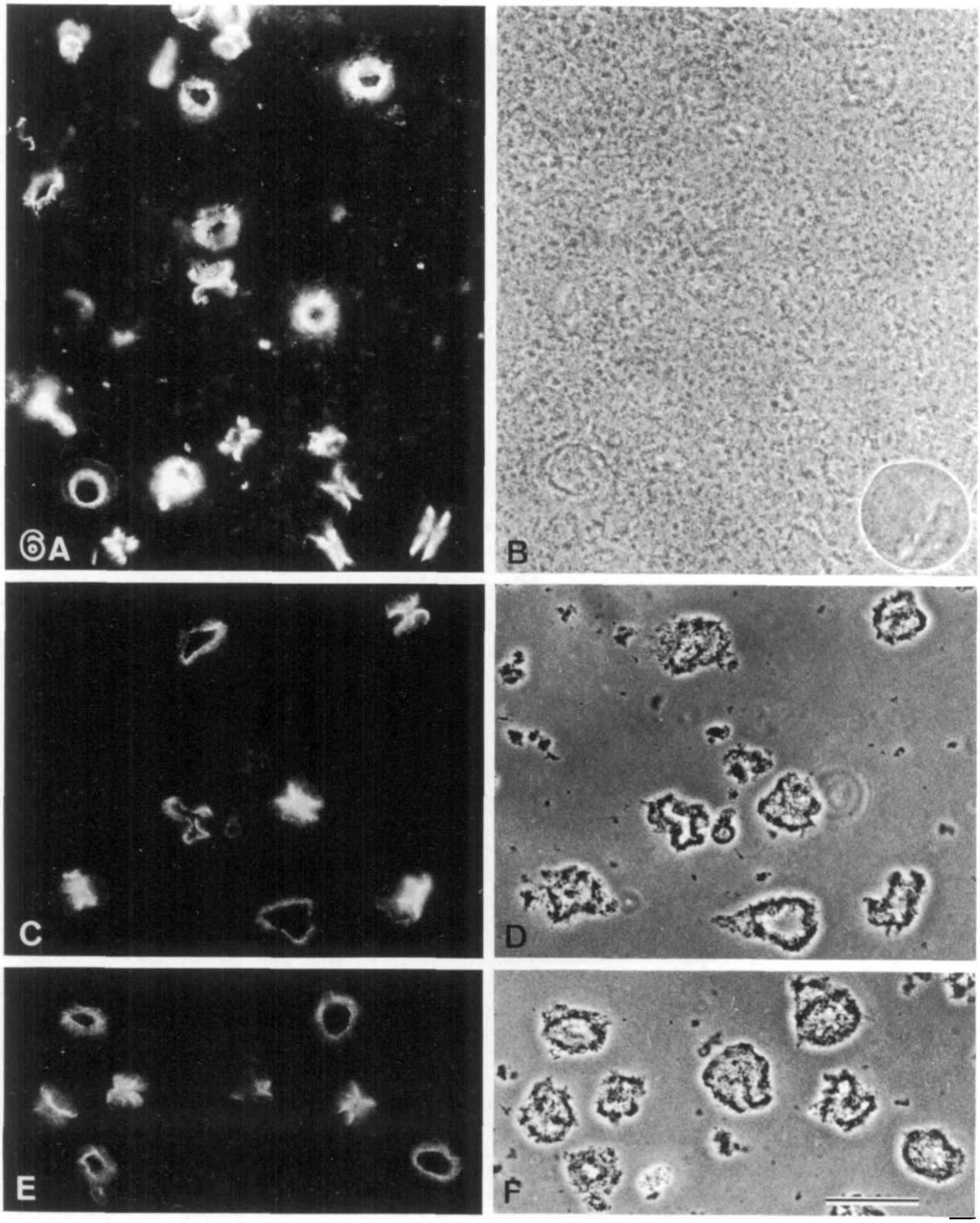

Fig. 6. Fluorescence (A,C,E) and phase-contrast (B,D,F) micrographs of cleavage furrows isolated from *C. japonicus* eggs. Dividing eggs were lysed and gently sheared in the isolation medium (A,B). (A) Cleavage furrows and small cortical fragments are seen to contain actin filaments. (B) These isolated furrows are surrounded by enormous amounts of cytoplasmic material. (C,D) Purified cleavage furrows obtained after sucrose gradient centrifugation. (E,F) Different specimen of purified cleavage furrow. Only a few cytoplasmic particles and cortical fragments are seen. Bar, 100 μm.

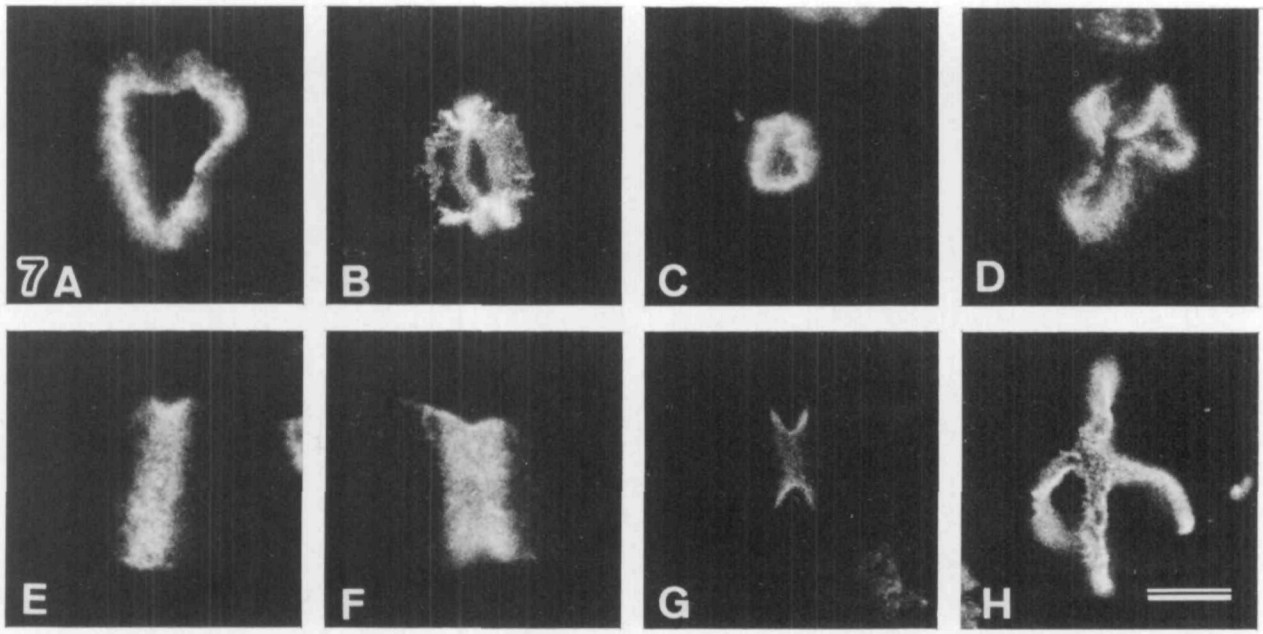

Fig. 7. Fluorescence images of cleavage furrows isolated from *C. japonicus* eggs stained with Rh-ph. (A) Frontal and (E) lateral views of isolated furrows that are large in diameter. (B) Oblique and (F) lateral views of furrows of medium diameter. (C) Frontal and (G) lateral views of isolated furrows that are small in diameter. (D) A furrow folded during isolation. (H) Two furrows joined at right angles, isolated from a polyspermic egg. Bar, 30 μm.

then added 2 mM MgATP to the furrow under various Ca^{2+} concentrations (0–100 μM). However, we have failed to detect any significant contraction of the isolated furrow under the present conditions. The same negative result was obtained with dividing eggs treated only with detergent, and with cortex, including the cleavage furrow, isolated on coverslips.

Discussion

To establish a mass isolation procedure for the cleavage furrow from synchronized sea urchin eggs, we have carefully analyzed the factors that might affect the stability of the cleavage furrows, the remainder of the cortices (the polar-region cortices) and the inner cytoplasm. The results obtained from these analyses were as follows: to remove the polar-region cortex and leave the cleavage furrow intact, the type and concentration of detergent, the pH and Ca concentration of the isolation medium and the temperature should be determined carefully. For four species of sea urchin eggs used in this study, 0.04–0.1% Nonidet P-40, pH 7.0–7.5, low calcium ion concentration and room temperature were optimal conditions. To solubilize the inner cytoplasm to release intact cleavage furrows, two factors (osmotic pressure of the isolation medium and sea urchin species) were important; 0.16 M glucose (or sucrose) was optimal, and *C. japonicus* was found to be the most appropriate for this purpose. A moderate shearing force by gentle pipetting was required both for peeling-off the polar-region cortex and for solubilization of the inner cytoplasm. Taking these results into consideration, we then successfully developed a mass isolation procedure for the cleavage furrow from *C. japonicus*. A total of 20–50 μg of protein from isolated cleavage furrow was recovered from 1 ml of packed dividing eggs. The isolated cleavage furrow maintained its structural integrity *in situ*, contained the contractile ring, and showed no capability of *in vitro* reactivation.

This paper is the first demonstration that the mass isolation of the cleavage furrow, including the contractile ring, is possible under appropriate conditions using sea urchin eggs. This achievement is mainly attributed to recent improvements in the specific staining method for actin filaments. Since the cleavage furrow is a transitory and labile structure, a quick method for its identification was required in order to develop the isolation procedure. Until recently, it had been very difficult to identify quickly the released cleavage furrows in the cell lysate, if the cleavage furrow and/or the contractile ring were to be successfully released from dividing cells. About 15 years ago, Schroeder (1975) pointed out the possibility of the isolation of cleavage furrow from sea urchin eggs using conventional light microscopy, but he failed to establish an isolation method at that time. In this study, taking advantage of the rhodamine-phalloidin staining method, the effects of many factor on the stability and morphology of the isolated cleavage furrow could be analyzed carefully.

The isolated cleavage furrow was characterized at the electron-microscopic level by remnants of plasma membranes, a layer of actin filament meshwork, and cytoplasmic vacuoles. Inside the actin filament meshwork, a layer of densely packed actin filaments was reproducibly identified. Its width (~0.2 μm), thickness (~4 μm) and center-to-center spacing of actin filaments (8–14 nm) were almost identical to those reported for the contractile ring *in situ* (Schroeder, 1972; Mabuchi, 1986), indicating that the structural integrity of the contractile ring was well maintained in isolated cleavage forrows. Interestingly, as shown in Fig. 8B, this contractile ring appeared to be tightly associated with, and occasionally continuous with, the surrounding actin filament meshwork. This image seems to favor the recent idea that the contractile ring may be formed from pre-existing cortical actin filaments

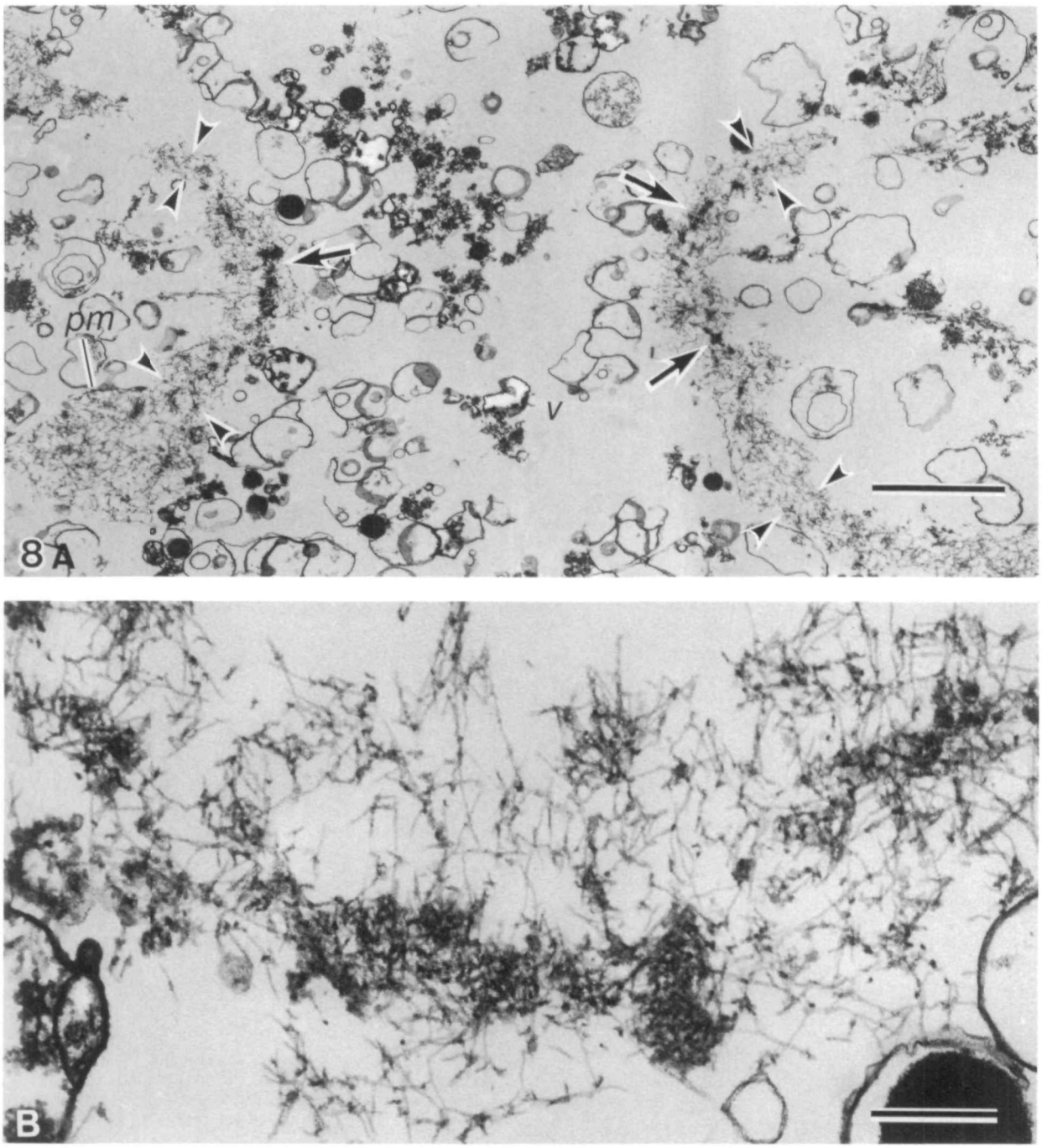

Fig. 8. Electron micrographs of a cleavage furrow isolated from a *C. japonicus* egg. A cross-sectional view of an isolated cleavage furrow at lower magnification (A) and at higher magnification (B) showing the contractile ring. The isolated furrow is composed of remnants of the plasma membrane (pm), a layer of actin filament meshwork (regions indicated by pairs of arrowheads), and cytoplasmic vacuoles (v). (B) At the bottom of the cleavage furrow (arrows in A), the contractile ring is identified as bundles of microfilaments. Bars: 2 μm (A); 0.3 μm.

(Cao and Wang, 1990). Although the morphology of the contractile ring was well maintained in isolated cleavage furrow, and, furthermore, isolated furrow was shown to contain myosin II molecule (see Fig. 9C,D), we have not yet succeeded in reactivating isolated cleavage furrow. In sharp contrast to this result, cleavage furrow isolated manually from newt eggs was recently reported to be reactivated by adding ATP (Mabuchi *et al.* 1988). Considering that in the case of newt eggs the cleavage furrow was isolated surgically without detergent treatment, the furrow isolated in this study probably requires for its contraction some soluble factors that could be lost during either the detergent treatment or the following isolation procedure. One candidate would be the myosin phosphorylation system, which has been reported to play an important role in motility in both non-muscle (Daniel *et al.* 1984; Berlot *et al.* 1985) and smooth muscle cells (Bennet *et al.* 1988).

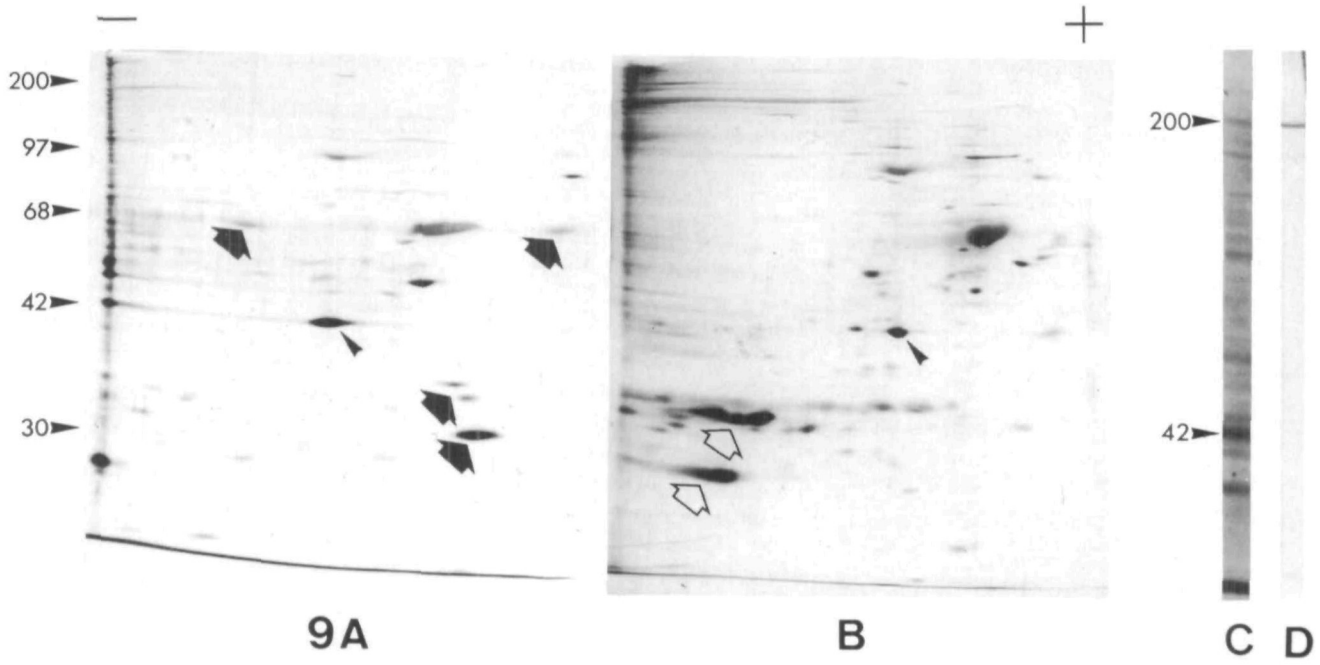

Fig. 9. Electrophoretic and immunoblot analyses of isolated cleavage furrows. Two-dimensional gel electrophoresis of the furrows (A) and of the egg cortices isolated at late prophase (B). Actin is indicated by arrowheads. Molecular weight markers are myosin heavy chain (200×10^3), phosphorylase *b* (97×10^3), BSA (68×10^3), actin (42×10^3) and carbonic anhydrase (30×10^3). Proteins unique to isolated furrows and to cortices are indicated by filled arrows and open arrows, respectively. (C) Total proteins in the furrow fraction electrophoresed, transferred onto a nitrocellulose membrane and then stained for protein. (D) Immunoblot analysis of the furrow fraction using anti-myosin II antibodies. A 200×10^3 M_r protein in the furrow fraction is specifically stained.

Another point we must discuss here is the problem that the efficiency and purity of the isolation of cleavage furrow are different from species to species. This may be mainly because the stability of the cleavage furrow from each species was different under the isolation condition used in this study. Among four species we examined, *C. japonicus* was the most appropriate for furrow isolation. However, considering that in the other three species cleavage furrow could be isolated, though at a lower efficiency and less pure, it may not be too difficult to find eggs appropriate for furrow isolation among various kinds of sea urchin that we did not examine in this study.

In summary, we developed a mass isolation procedure for cleavage furrow using dividing sea urchin eggs. This procedure should give us many clues towards understanding the molecular mechanism of cytokinesis. One of the promising approaches using isolated furrows is to analyze the furrow-specific proteins carefully (see Fig. 9A,B). Monoclonal antibody production against isolated furrows should be attempted. Searching for factors that make the reactivation of isolated furrows possible should lead us to a better understanding of the regulation mechanism of cytokinesis. Studies along these lines are now being carried out in our laboratories.

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