Tubulin polymerization in unfertilized sea-urchin eggs induced by elevated temperature

PATRICIA J. HARRIS, ELISE L. CLASON and KEVIN R. PRIER
Department of Biology, University of Oregon, Eugene, OR 97403, USA

Summary
Spontaneous polymerization of tubulin was induced in unfertilized eggs of the sea urchins Lytechinus pictus and Strongylocentrotus purpuratus by warming to temperatures 10-12 deg. C above the normal environment for each species. Indirect immunofluorescence microscopy showed that over a period of several hours polymerization proceeded from a fine crystal-like precipitate to larger and fewer higher-order structures. These structures differed morphologically between the two species: L. pictus formed cytasters, while S. purpuratus most often formed flame-like arrays. Nuclear cycles were not initiated, permitting long-term observation of steady-state polymer redistribution. After several hours only a few very large cytasters remained near the surface of L. pictus eggs and these tended to constrict to form protrusions or occasionally a cleavage furrow that divided the egg into two fragments. Staining with rhodamine-labeled phalloidin showed that actin was also concentrated in the cytasters, primarily at the periphery of the centrosphere-like regions.

L. pictus cytasters could be recycled by repeated cooling and rewarming, and showed an accelerated rate of polymerization with each successive cycle. Cytasters could also be isolated as long as the initial procedures were carried out at the elevated temperature. Washed preparations were stable at room temperature. From these observations we conclude that: (1) species-specific cytoplasmic factors control the temperature limits for spontaneous tubulin polymerization in unfertilized eggs, as well as the form of higher-order structures that are produced; (2) tubulin polymerized in whole eggs at elevated temperature shows characteristics similar to purified tubulin in vitro, with regard to cold recycling and steady-state polymer redistribution; (3) actin also polymerizes at the elevated temperature and is found closely associated with microtubules of the cytasters.

Key words: microtubules, unfertilized sea-urchin eggs, cytasters.

Introduction
Although microtubules are known to exist in immature oocytes of various invertebrates (e.g. see Otto & Schroeder, 1984), they are generally thought to be absent in unfertilized mature sea-urchin eggs. At normal physiological temperature no microtubules are visible with immunofluorescence techniques (Harris et al. 1980; Bestor & Schatten, 1981), nor have they been reported in any electron microscopy studies. Unfertilized eggs contain a large pool of soluble tubulin (Raff & Kaumeyer, 1973; Pfeffer et al. 1976), but assembly is prevented by some inhibitory factor. The presence of an inhibitor was believed to be responsible for difficulties in obtaining assembly-competent tubulin from unfertilized sea-urchin egg extracts, but recently Suprenant & Marsh (1987) have shown that standard procedures for mammalian brain tubulin carried out at 37°C are not applicable to an organism whose upper temperature limit is 25°C (Farmanfarmaian & Giese, 1963). Spontaneous assembly of microtubules was obtained in unfertilized Strongylocentrotus purpuratus egg extracts warmed to 24°C, about 12°C above normal environmental temperature, but not to temperatures above 28–30°C (Suprenant & Marsh, 1987).

We have examined the effect of elevated temperature on tubulin polymerization in living unfertilized eggs of Lytechinus pictus and Strongylocentrotus purpuratus and found that temperatures 10–12 deg. C above normal environmental temperature for each species will induce microtubule polymerization without causing immediate cell death. Not only are microtubules assembled, but they are organized into higher-order structures that are characteristic for each species. We describe here some of the properties of these structures and their relevance to normal cellular functions.
Materials and methods

Gametes

Eggs and sperm of the sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* were obtained by injection of 0.5 M-KCl into the body cavity. Eggs were washed and held at 17°C for *L. pictus* and 12°C for *S. purpuratus* (normal temperature for each species) until used. Batches of eggs were test-fertilized before the experimental procedures.

Incubation

Eggs were collected by gentle hand centrifugation and warmed rapidly by resuspension in artificial sea water prewarmed to 30°C for *L. pictus* and 24°C for *S. purpuratus*. These temperatures were slightly above the minimum required for tubulin polymerization as determined in previous experiments. Samples of eggs were removed at intervals during a period of approximately 5 h for fluorescence microscopy or fixation for embedding, and living eggs were observed for at least 24 h.

Fluorescence microscopy

Drops of egg suspension were placed on polylysine-coated coverslips previously brought to the appropriate temperature within a Petri dish moist chamber floating on the water bath. After several minutes to allow the eggs to settle, coverslips were transferred rapidly to the appropriate fixative. Speed was important during this transfer, as any drop in temperature could result in microtubule disassembly.

For tubulin immunofluorescence, coverslips were fixed for 6 min in -20°C methanol containing 25 mM-EGTA at pH 6-0, rehydrated in phosphate-buffered saline (PBS) and incubated at 37°C for 2-4 h or overnight with a rat monoclonal antibody (YL1/2) against tyrosinated alpha tubulin. This antibody, when compared with a rabbit polyclonal antibody against sea urchin egg tubulin (Dako, Santa Barbara, USA), showed identical localization within the limits of resolution of our preparations. The choice was based on slightly better staining obtained with the YL1/2 and antibody availability. Eggs were then washed three times (5 min) in PBS and incubated for 2-4 h in rhodamine-labeled goat anti-rat IgGs (Cappel). The coverslips were washed four times in PBS, with the first wash containing Hoechst 33258 stain for DNA, and then mounted on slides with Mowiol (Osborn & Weber, 1982).

For actin localization, eggs on coverslips were fixed in 1% paraformaldehyde in 0.43 M-sodium acetate, pH 6-1, at 30°C for 20 min, washed in PBS and stained for 20 min with rhodamine-labeled phalloidin (Molecular Probes, Inc., Eugene, OR, USA). The coverslips were washed briefly in PBS and mounted with Mowiol. Photographs were made on Kodak Tri-X film, using a Zeiss Universal microscope equipped for epifluorescence.

Cytaster isolation

Eggs were incubated at 30°C in calcium-free artificial sea water. After 60-90 min they were re suspended at 30°C in 1 M-hexylene glycol in 0.01 M-phosphate buffer at pH 6.4 (Kane, 1965). After 1-2 min the solution was shaken vigorously to break the cells and release the cytasters. Cytasters were washed by repeated pelleting and resuspension in fresh isolation medium.

Stereo micrographs

A Zeiss lens mount for differential interference contrast microscopy was used with a 25X Neofluor oil immersion lens. In place of the slider with prism, a blank slider with its circular opening half-covered was used for the pair of photographs by inserting the slider first from one direction and then the other (for details, see Osborn & Weber, 1979).

Note that indicated magnification may seem variable as a result of flattening of eggs on the polylysine surface, or from shrinkage or swelling caused by fixation procedures. Approximate size of freshly shed eggs is 100 μm for *L. pictus* and 75 μm for *S. purpuratus*.

Results

The pattern of tubulin polymerization

The early stages of tubulin polymerization in *L. pictus* and *S. purpuratus* were very similar, beginning with what appeared to be a fine, almost crystalline precipitate distributed uniformly throughout the cell. As polymerization continued, larger structures developed, which differed in the two species. *L. pictus* formed cytasters, while in *S. purpuratus* the structures that formed were more variable. Flame-like bands were most common, and these elongated over time. In other batches of eggs, however, networks of microtubules, often in conjunction with the flame-like structures, appeared near the cell surface (Fig. 1), and on rare occasions an individual cell developed small cytasters.

*L. pictus* was more consistent and almost always produced well-formed cytasters (Figs 2-6). After about 45-60 min the total amount of polymerized tubulin appeared to reach a peak, with all tubulin fluorescence present in formed structures and very little background fluorescence in the cytoplasm. Continued incubation at the higher temperature brought about a redistribution of polymerized tubulin that resulted in the further growth of large structures at the expense of smaller ones.

The nuclear cycle was not initiated by the elevated temperature or by the presence of polymerized tubulin, an important characteristic shared by both species. Unlike cytasters in artificially activated eggs, where the nuclear cycle causes periodic breakdown and regrowth of microtubule structures, polymer redistribution could be observed over periods much longer than a normal division cycle.

The pattern of individual cytaster growth in *L. pictus* was similar to that of normal mitotic asters, especially in eggs of various species. In the earliest stages microtubules originated from the aster center, but as total aster size increased, an empty central region, resembling a centrosphere, appeared and also grew in size. The series of stereo micrographs (Figs 2-6) shows the progressive aster development in *L. pictus* from 30 min to 120 min. As aster size increased, the number of cytasters decreased, apparently by the breakdown of the smaller ones. We never observed a direct merging of two cytasters. After several hours many eggs had only two or three cytasters attached to the cell surface. The nucleus, also at the cell surface, was located at the center of an aster (see Figs 7 and 8).

Cytasters and the presence of actin

The greatly enlarged asters were held at the cell surface at the point of contact of the outer rim of the centrosphere-like region and the egg cortex, thus flattening the aster on
one side and giving it the appearance of a fringed ring (Fig. 6). Whole eggs fixed in paraformaldehyde and stained with rhodamine-labeled phalloidin showed that filamentous actin was present in these ring-like asters at the egg periphery (Fig. 7). The greatest concentration of actin appeared to be at the rim of the central region, but additional bands separated adjacent asters. In addition to the staining associated with the asters, nuclei were also stained with the rhodamine-phalloidin (Figs 7 and 8). Subsequent examination of earlier stages showed that

Fig. 1. *S. purpuratus* egg warmed to 24°C for 60 min. Indirect immunofluorescence staining for tubulin shows microtubule networks that may appear along with the more common bands of flame-like structures. Stereo micrographs are mounted for proper depth perception when viewed with a 2X stereo viewer. Bar, 50 µm.

Fig. 2. Unfertilized *L. pictus* egg warmed to 30°C for 30 min, showing finely dispersed polymerized tubulin throughout the cytoplasm, similar to that seen in *S. purpuratus* after warming to 24°C for 30 min. Bar, 50 µm.

Fig. 3. Another egg from the same 30 min sample as shown in Fig. 4, showing an early stage of cytaster formation. Bar, 50 µm.
even when cytasters were deep within the cytoplasm, actin had colocalized with the tubulin. Fig. 8 shows an extremely flattened egg stained with rhodamine-phalloidin at 60 min, with cytasters clearly visible.

The exact relationship between the actin and the microtubules in these structures could not be determined at this time, so we cannot say whether the actin and tubulin rings actually coincide or are adjacent. Owing to
Fig. 7. Rhodamine-labeled phalloidin staining for filamentous actin shows localization near the periphery of the centrosphere of greatly enlarged cytasters after 120 min at 30°C. Another band of actin appears to separate the three asters. Note that the nucleus is also stained and is surrounded by one of the aster rings. Compare with tubulin localization in Fig. 6. Bar, 50 μm.

Fig. 8. A highly flattened egg stained with rhodamine-phalloidin after 60 min at 30°C. Actin is clearly localized in cytasters throughout the egg as well as around the nucleus. Compare with a similar cell stained for tubulin in Fig. 4. Bar, 50 μm.

Fig. 9. Freshly shed *L. pictus* eggs, showing the primarily cortical position of the nuclei. Bar, 100 μm.

Fig. 10. Nuclei become centered within 30 min in eggs initially warmed for no longer than 60 min and then cooled to 17°C. This centering is lost after several hours at the lower temperature. Bar, 100 μm.

Fig. 11. A stained section of an egg warmed to 30°C for 60 min and cooled to 17°C for 30 min. Long microtubules are associated with the nucleus and form a meshwork throughout the egg, but no formed cytasters are seen. Bar, 10 μm.

**Effect of cooling previously warmed eggs**

As long as eggs remained at the elevated temperature, they generally retained their spherical shape, but on cooling changes in the cytoskeleton structure often led to cell deformation. The degree of deformation apparently depended on the number of cytasters remaining, and how closely they were applied to the cell surface.

If eggs were cooled to 17°C before the cytasters became closely associated with the egg cortex (about an hour), nuclei became exactly centered within 30 min. Fig. 9 shows the normal cortical position of nuclei in freshly shed eggs. Compare with centered nuclei in eggs cooled for 30 min (Fig. 10). After several hours of recovery at 17°C, however, nuclear position in the eggs became random. To determine whether microtubules were in-

**Extreme fragility of the eggs with this fixation, adequate double staining for tubulin and actin were not obtained before the end of the urchin spawning season. However, compare eggs stained separately for tubulin and actin at 120 min (Figs 6 and 7) and at 60 min (Figs 4 and 8).**
When eggs are held for 3–4 h at 30°C and then cooled, the egg surface constricts at the site of the aster rings, resulting in a protrusion of the aster centers. Bar, 100 μm.

After several hours of cooling the eggs begin to round up and the protrusions subside, leaving in their place puckered dimples on the egg surface. Bar, 100 μm.

When only one cytaster remains to girdle the cell, constriction produced by cooling may result in a cleavage furrow (arrows) that separates the egg into a smaller nucleated and a larger enucleated fragment.

After several hours recovery at 17°C these eggs have regained their spherical shape. Two eggs have divided. Arrows indicate completed cleavage furrows. Bar, 100 μm.

If eggs at later stages with greatly enlarged cytasters attached to the cortex were cooled to 17°C, nuclear centering did not occur. Instead, the egg surface became constricted at the site of the aster rings, resulting in a protrusion of aster centers (Fig. 12). After several hours the protrusions were resorbed, often leaving puckered dimples on the egg surface (Fig. 13), and suggesting but not proving that the aster rings had closed. In a few cases (1–5%), where only one remaining large aster girdled the egg, this constriction produced a cleavage furrow, visible as a light band in Fig. 14. This ring also closed, but the

Recycling of cytasters by cooling and rewarming

To test the possibility that elevated temperature caused irreversible changes in the tubulin, eggs incubated at 30°C for 60 min were cooled rapidly to ice temperature for 60 min and then returned to 30°C. The results of three polymerization cycles from one of several different batches tested are shown in Fig. 16. Vertical columns...
Fig. 16. Cytasters could be recycled, as shown here in three polymerization cycles (vertical columns I, II and III). Cycle I: A. Untreated unfertilized eggs; B, 30 min at 30°C; C, 60 min at 30°C; cycle II: D, 60 min at 0°C; E, 30 min at 30°C; F, 60 min at 30°C; cycle III: G, 60 min at 0°C; H, 30 min at 30°C; I, 60 min at 30°C. Bar, 50 μm.
show cycles I, II and III before warming and after 30 min and 60 min at 30°C. Each successive cycle polymerized microtubules more rapidly than the preceding one. For example, polymerization in the 30 min sample in cycle II is much more advanced than in the 60 min sample in cycle I. Although the cytasters disappeared, apparently not all the tubulin was depolymerized after 60 min in the cold, as seen by scattered tubulin immunofluorescence in the cold-treated eggs.

**Fertilization and development of warmed eggs**

Eggs of *L. pictus* were test-fertilized to determine their viability following extended periods at the elevated temperature. When warmed sperm were added to eggs held at 30°C for 90 min fertilization membranes rose, indicating at least some degree of activation. Examination of sections of paraformaldehyde-fixed eggs showed partial breakdown of large cytasters at 15 min after fertilization and their complete loss after 60 min, with only a fine network of microtubules remaining. Living eggs remaining at the higher temperature showed no further signs of development.

When eggs were held at 30°C for 90 min and then cooled to normal temperature for 60 min before adding sperm, fertilization membranes also formed readily. Sectioned material showed that after 15 min large cytasters still remained, with no apparent sperm aster. Attempts at division were not normal, resulting after several hours in aggregates of cells of various sizes and sometimes amoeboid shapes. There was no further development.

**Isolation of cytasters**

Cytasters could be isolated from warmed eggs as long as initial operations were carried out at the elevated temperature. Once the eggs were broken and the cytasters released into the isolation medium, the cytaster structure was stabilized. Washing by repeated centrifugation and resuspension in isolation medium could be carried out at room temperature. The method appears useful for structural and biochemical studies of the cytasters from unactivated eggs, but we did not carry the work further at this time.

**Discussion**

**Patterns of tubulin polymerization**

Tubulin polymerization in warmed unfertilized *L. pictus* and *S. purpuratus* eggs resembled in some respects the taxol-induced cytaster formation in *Arbacia punctulata*, obtained by Schatten et al. (1982). The eggs were not activated and nuclear cycles of breakdown and re-formation did not occur. Taxol induces free microtubule polymerization *in vitro* by lowering the critical concentration of tubulin (Schiff et al. 1979). Increasing temperature also lowers the critical concentration in preparations of mammalian brain tubulin (Gaskin et al. 1974). Very probably the elevated temperature acts in a similar way in the unfertilized eggs. However, it is possible that the increased temperature may release inactivated monomer to raise the concentration of the monomer pool and thus promote microtubule assembly, but these experiments do not distinguish between the two mechanisms.

Although the earliest stages of tubulin polymerization were similar in *L. pictus* and *S. purpuratus*, the type of structures formed soon diverged, reflecting the greater tendency of *L. pictus* eggs to form cytasters after artificial activation (Kuriyama & Borisy, 1983). As the cytasters in *L. pictus* provided a better system for estimating numbers and sizes of structures, the emphasis was on this species, leaving *S. purpuratus* for further investigation.

The transition from many small cytasters to fewer and larger ones followed, at least superficially, a pattern similar to that of steady-state microtubule dynamics reported by Kristofferson et al. (1986), where average length increased and number of microtubules decreased. By analogy, in the sea-urchin eggs at steady state continuous monomer/polymer exchanges, possibly mediated by the mechanism of dynamic instability, provided a supply of unpolymerized tubulin. The ultimate site of repolymerization was probably that which provided the most stable state, in this case the largest structure.

The progressive changes in cooled eggs that resulted in nuclear centering might also be the result of polymer redistribution. Bundles of long microtubules, usually associated with the nucleus, replaced the depolymerizing cytasters, but it is not at all clear how the changes in microtubules bring about the centering. The lack of centering in similar eggs that are cooled rapidly to 0°C probably reflects the more rapid loss of microtubules.

**Actin localization in the cytasters**

Actin, like tubulin, responds to increased temperature with a lowering of the critical concentration, thus shifting the monomer/polymer equilibrium toward polymerization (Oosawa & Asakura, 1975). It is therefore not surprising that actin would also polymerize under our experimental conditions. Although some form of polymerized actin has been detected biochemically in unfertilized eggs at normal temperature (Otto et al. 1980; Coffe et al. 1982), it is only recently that improved fixation methods for electron microscopy and fluorescence probes have revealed fibrous meshworks within the short microvilli and a non-filamentous form localized in the cortex (Henson & Begg, 1988; Spudich et al. 1988). Filamentous actin has also been found within nuclei of unfertilized eggs (Spudich et al. 1988), similar to our observations on warmed eggs.

While microtubules do not appear associated with actin in the cortical structure of mature unfertilized sea-urchin eggs, some interaction between actin and tubulin occurs during the monaster cycles of activated eggs. For example, studies on cytoplasmic cohesiveness cycles in artificially activated sea-urchin eggs (Coffe et al. 1982, 1983) have shown that while the cycle itself is regulated by tubulin polymerization and depolymerization cycles, the increase in cytoplasmic cohesiveness requires the polymerization of both tubulin and actin. Our finding of a selective polymerization of actin within or onto the tubulin cytaster scaffold provides further evidence for a
Relevance to normal cellular function

Recycling of cytasters by repeated cooling and rewarming, and fertilization of eggs at the elevated temperature as well as after a period of cooling, were carried out to demonstrate that the system was not irreversibly damaged. While the eggs responded with raising of fertilization membranes, abnormal divisions resulted, probably as much from the geometry of dividing a cell filled with cytasters as from any physiological impairment. These results indicated that while the warmed eggs were close to the limit of viability, they were not dead. They could still respond to external stimuli and they retained their species-specific temperature sensitivity and drug perturbations.

Certain similarities between the results of our experiments and normal cellular functions offer insight into the mechanisms of these functions, and should be investigated more directly. For example, how much of the pattern formation of tubulin polymerization (aster shape, formation and breakdown) is determined by tubulin polymerization kinetics itself? How is the hierarchy of polymerization sites determined during polymer redistribution? Does this play a role in the transition of one aster system to another? Finally, an old question, what is the interaction between asters and the cell cortex that induces a furrow?

This work was supported by grant PCM-8409573 from the National Science Foundation to P.J.H.

References


(Received 3 November 1988 – Accepted 18 January 1989)