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Evidences for Direct Involvement of Microtubules in Cleavage Furrow Formation in Newt Eggs

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ABSTRACT—This paper aims at examining the effect of colchicine, a microtubular poison, on the process of furrow formation in whole eggs and egg fragments as well as the process of artificial induction of furrow-like dents, in eggs of the newt, *Cynops pyrrhogaster*. To apply colchicine locally to eggs, the eggs were slit across or along a furrow in a colchicine solution during first cleavage. When a slit was made across or in front of a growing furrow at the onset of its growth, the furrow quickly ceased growing and often regressed. Cortices containing an entire growing furrow were isolated along with a thin layer of subcortical cytoplasm immediately after the start of the first cleavage. Furrows in the cortices degenerated when the cortices were cultured in a colchicine solution, whereas they continued growing when they were cultured in Holtfreter's saline. Furrow-inducing cytoplasm was injected to a site beneath the cortex in the animal half of the egg during first cleavage. When a small slit was made close to the site of the injection in a colchicine solution, no furrow-like dent was induced. These results imply that microtubules are directly involved in the generation and growth of cleavage furrows.

INTRODUCTION

Studies on cytokinesis in a variety of animal cells and eggs have demonstrated that a cleavage furrow is always formed on the cortex between the two asters of the mitotic apparatus (MA) (reviews by Conrad and Rappaport,1981; Mabuchi, 1986; Rappaport, 1971, 1986; Schroeder, 1981). Observations with electron microscopy have shown that a bundle of microfilaments, mainly composed of actin filaments, is aligned immediately beneath the furrow (Schroeder, 1968; Arnold, 1969; Tilney and Marsland, 1969; Bluemink, 1970; Selman and Perry, 1970; and reviews by Schroeder, 1975, 1981; Mabuchi, 1986; Mabuchi and Itoh, 1992). It is broadly accepted that myosin is involved in the formation and contraction of the furrow (Fujiwara and Pollard, 1976; Mabuchi and Okuno, 1977; Schroeder and Otto, 1988; and reviews by Schroeder, 1981; Mabuchi, 1986; Mabuchi and Itoh,1992). On the basis of these facts, an explanation has been proposed for the mechanism of cytokinesis in animal cells: A stimulus generated in the central region of the egg, mediated by the MA, reaches the region of the cortex on the presumptive cleavage plane and induces a ring or an arc of bundles made of mainly actin fibers and myosin to form there.

There are many studies on the process and stage of cell division at which the MA operates to achieve cell division. In echinoderm eggs, displacement (Hiramoto, 1956; Rappaport and Ebstein, 1965; Rappaport, 1997), removal (Hiramoto, 1956, 1965,1971) or disintegration (Hamaguchi, 1975; Hiramoto, 1965) of the MA at the anaphase of mitosis did not affect cleavage-furrow formation, while those before metaphase changed the cleavage plane or caused the failure of furrow formation. Similar results have been obtained in amphibian eggs (Kubota, 1966; Sawai and Yomota, 1990; Selman, 1982; Waddington, 1952; Zotin, 1964). From these results, it is thought that in cytokinesis the MA determines the cleavage plane at the anaphase of mitosis, or shortly before the generation of a visible furrow, by transferring a stimulus to the cortex, and once the cortex has received the stimulus it can form a furrow without the MA. In other words, the MA is not necessary for the formation or deepening of a furrow.

We have demonstrated that subcortical cytoplasm on a cleavage plane of amphibian eggs induces furrow-like dents when transplanted to a subcortical region outside the plane (Sawai, 1972, 1983; Sawai *et al*., 1969). This indicates that subcortical cytoplasm on a cleavage plane contains a factor responsible for furrow formation, i.e. furrow-inducing cytoplasm (FIC). We have shown further that during cleavage the cortex of amphibian eggs acquires an ability to form a furrow in response to the inductive activity of the FIC (Sawai, 1972, 1974, 1983). Cleavage furrows of amphibian eggs are thus formed by the interaction between the cytoplasmic factor (FIC) and some cortical factor.

In amphibian eggs, colchicine inhibited the generation of a cleavage furrow when injected into eggs before the onset of cleavage (Sawai and Yomota, 1990; Sentein, 1979; Sentein and Atès, 1978) and its growth when injected after that (Kubota and Sakamoto, 1993; Sawai and Yomota, 1992). The latter

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fact suggested that microtubules participate in the birth and growth of cleavage furrows. In a previous paper (Sawai, 1992), the involvement of microtubules in furrow formation was tested by examining the effects of microtubular poisons on the formation of a furrow in normal cleavage and on the induction of furrow-like dents by FIC transplantation, and it was shown that microtubule involvement is likely. To corroborate these previous conclusions, the present study examines closely the effect of colchicine on furrow formation in newt eggs using three experimental systems, i.e. furrow formation in normal cleavage and that in isolated egg fragments, and the induction of furrow-like dents by FIC.

MATERIALS AND METHODS

Eggs of the newt, *Cynops pyrrhogaster*, were used exclusively in the experiments. Fertilized eggs were obtained by hormonal stimulus with chorionic gonadotropin. About 80 IU of the hormone was injected every other day into the abdomen of female newts that had gained spermatophores in their habitat.

Eggs were deprived of the jelly capsule by treatment with 1.5 % sodium thioglycollate solution (pH 10.0). The vitelline membrane was removed with forceps. Denuded eggs were placed one by one in small depressions on an agar bed under Holtfreter's saline containing colchicine at a concentration of 0 or 1.0 mM. Making a slit and isolating a cortical layer were done manually with a fine glass needle. Injection of cytoplasm (about 100 nl) was done with a capillary with a diameter of about 50 μm (Sawai, 1972). The capillary was inserted into donor eggs during cleavage. After its tip was moved close to the surface of the plane of a furrow, subcortical cytoplasm was withdrawn into the capillary. The capillary was then pulled out and inserted into recipient eggs, and the cytoplasm of the donor eggs was injected to a site beneath the cortex opposite to that of the insertion of the capillary.

RESULTS

Wound healing on cleavage plane

The cortex of eggs during first cleavage was slightly slit across the cleavage furrow in Holtfreter's saline, and wound healing and the subsequent progress of the cleavage were observed. Figure 1 shows a typical result in eggs which were slit in front of the two ends of the furrow that has just started growing. Immediately after slitting, the furrow shrank rapidly so that the slits were made to open wider with their shape becoming rhombic. The wound from the slitting was completely healed after 2-5 min, with small scars left on the surface. Cleavage progressed without trouble thereafter. Similar results were obtained when slitting was done at other points on a furrow. These observations imply that the contractile structures along a furrow are tightly bound to the cortex of the cleavage plane and have contractility along the entire furrow plane. When a part of a furrow was removed, the wound from the removal surgery was closed along the cleavage plane, and the closed wound was changed to a deepening furrow (Fig. 2).

Effect of colchicine on furrow formation

To apply colchicine locally to eggs, slitting as shown in Fig. 1 was done at two points on the surface of a cleavage furrow in eggs placed in a colchicine solution. The slits closed

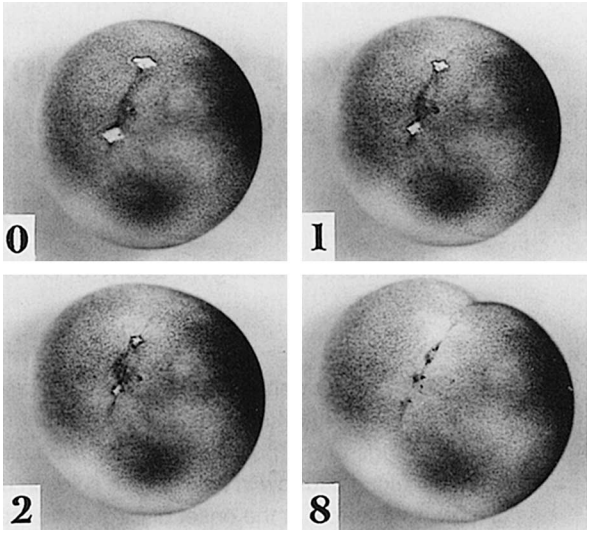

Fig. 1. Wound healing on a cleavage furrow. A slit was made at two points on the cleavage plane. The wound was rapidly closed and cleavage progressed normally. Numerals indicate the time in minutes after slitting. ×13.

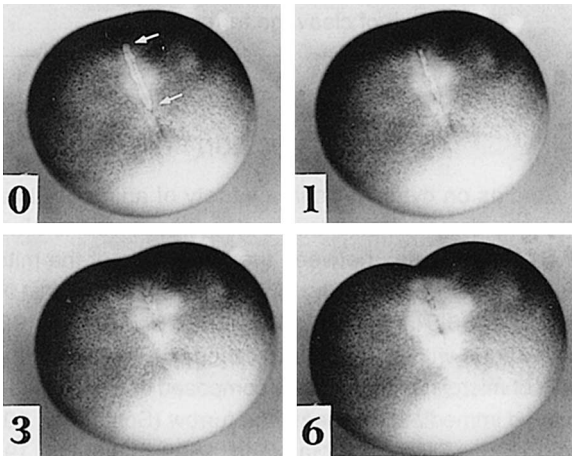

Fig. 2. Wound healing at a site where a part of the furrow was removed (between the two arrows). The wound was closed quickly along the cleavage plane (0-3 min) and the portion of slitting became a deepening furrow (6 min). Numerals; same as in Fig. 1. ×13.

in a similar fashion to that made in Holtfreter's saline. This simple operation allows the microtubule poison to permeate into a confined region in the eggs within a few minutes. Such a local treatment with a colchicine solution caused severe damage on the cleavage-furrow formation of operated eggs. The furrow usually degenerated immediately after rapid shrinkage between the two slits (Fig. 3). The subsequent process of the cleavage in these eggs was followed in view of several points, which were categorized into the following three groups: I. In cases in which the eggs recovered after the damage by colchicine, cleavage resumed 40-60 min after the degeneration of the furrow and was completed (6 of 17 cases). II. The furrow reappeared on the surface of the equator, skipping over

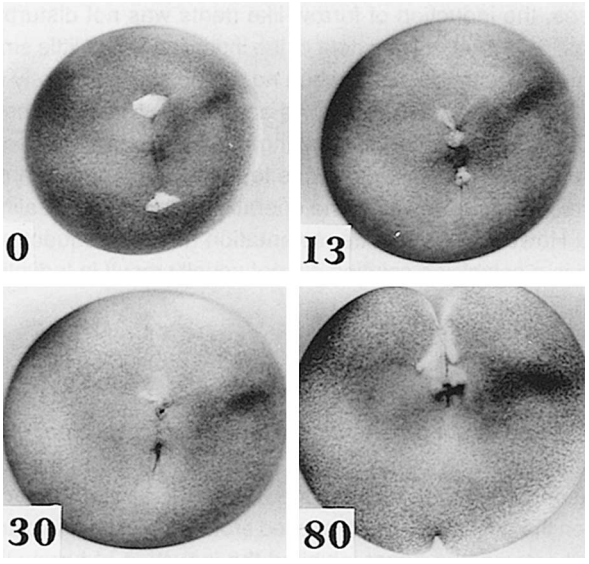

Fig. 3. A typical case of wound healing on the cleavage plane and the inhibition of furrow progress in a colchicine solution. The egg was slit at two points across the furrow in a colchicine solution and cultured there. The wound healed in a similar fashion to the eggs shown in Fig. 1, but the subsequent cleavage was disturbed. The furrow degenerated immediately after rapid shrinkage (0-30 min), and reappeared on the surface of the equator, resulting in a partial cleavage (80 min). Numerals; same as in Fig. 1. ×13.

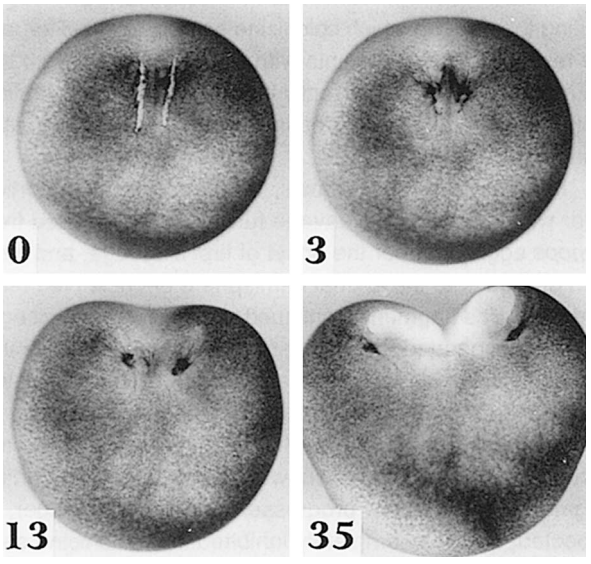

Fig. 4. A typical case of inhibition of cleavage in a case in which slits were made along a cleavage furrow. The furrow regressed just after slitting (0-13 min) and reappeared on one side of the equator (35 min). Numerals; same as in Fig. 1. ×13.

the degenerated area, and resulted in a partial cleavage as shown in the last print of Fig. 3 (9 of 17 cases). III. Cleavage was completely arrested (2 of 17 cases). It seems that the result may be influenced by the extent to which colchicine solution can permeate into the eggs. Similar results were obtained when slitting was done along the cleavage plane (Fig. 4). The number of the individual categorized cleavage patterns was 10/18 for complete cleavage, 6/18 for partial cleavage and 2/18 for arrested cleavage. When slitting was done at an advancing tip of a furrow halfway through the first cleavage, the number of categorized cleavage patterns observed was 14/22 for complete cleavage, 8/22 for partial cleavage and 0/22 for arrested cleavage. When slitting was done at a point more than 0.4 mm away from the cleavage plane, cleavage was little affected. This reveals that colchicine locally affected cleavage-furrow formation. When slitting was done around the center of a deepening furrow halfway through the first cleavage, the growth of the furrow was only slightly damaged.

As a control experiment, we previously reported that the cleavage of amphibian eggs suffered no damage when the eggs were cultured in a solution containing colchicine at concentration of 2.5 mM (1.0 mM in the present experiment) during the first cleavage cycle (Sawai and Yomota, 1992).

These results indicate that colchicine severely affected the establishment of a furrow and the stabilization of a nascent furrow, but not the deepening of a stabilized furrow.

Effect of colchicine on furrow progress in isolated egg fragments

Fragments of the cortical layer were isolated from the animal hemisphere of eggs just after the onset of first cleavage along with a freshly generated furrow. The fragment had been already prepared for furrow formation and could separate into two parts when it was transplanted to another egg (Sawai, 1980). In the present experiment, the fragment rapidly changed its shape to a round one within several minutes of isolation and continued to divide when kept on an agar sheet in Holtfreter's saline (Fig. 5A). In a colchicine solution, how-

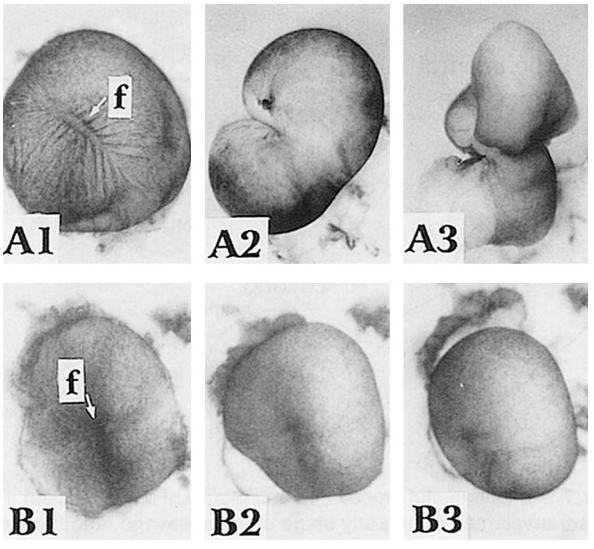

Fig. 5. Progress and failure of cleavage in a cortical fragment with a small furrow (f) in Holtfreter's saline (**A**) and a colchicine solution (**B**). **A1**, **A2** and **A3**; just, 20 and 30 min after the isolation. **B1**, **B2** and **B3**; just, 3 and 8 min after the isolation. ×21.

ever, the furrow of the fragment regressed quickly after isolation (Fig. 5B).

Effect of colchicine on the induction of a furrow-like dent

As a control experiment in Holtfreter's saline, portions of furrow-inducing cytoplasm (FIC) obtained from cleaving eggs were transplanted to a site beneath the animal cortex outside the cleavage plane immediately after the onset of first cleavage. Slits were made on the cortex at one or two of the sides of the transplantation site at two different stages; just after FIC transplantation and at the stage of pigment accumulation which is the first sign of induction of a furrow-like dent. In these cases, the induction of furrow-like dents was not disturbed (Figs. 6, 7) though the extent of the induction was a little small compared with the cases when no slitting was previously reported (Sawai, 1972, 1983). As another control experiment, FIC was transplanted to cleaving eggs in a colchicine solution, but with no slitting. In this test, the dent induction occurred in a similar way to the operation in Holtfreter's saline.

However, the FIC transplantation and subsequent slitting in a colchicine solution did not usually result in induction (Fig. 7).

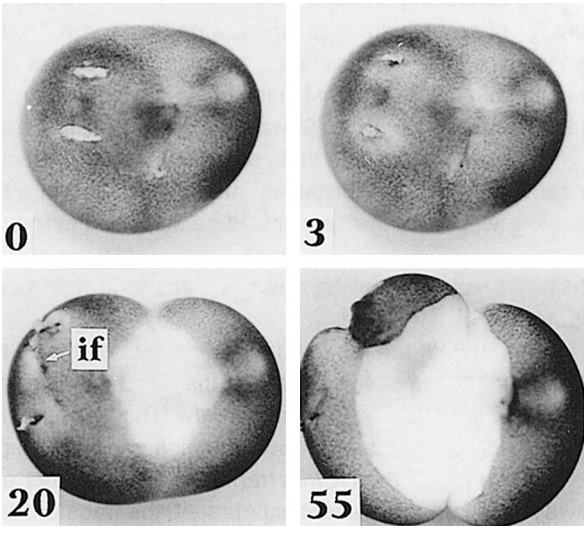

Fig. 6. Effect of slitting on the induction of furrow-like dents by FIC transplantation. FIC was transplanted to beneath the cortex and two slits were made at both sides of the transplantation about 10 min after transplantation. Dent induction occurred (if). Numeral; time (min) after slitting.

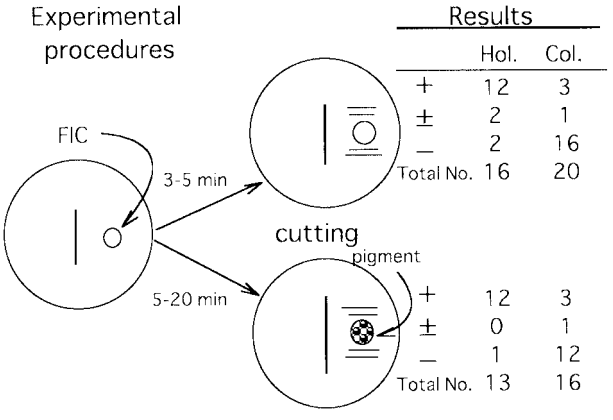

Fig. 7. Experimental procedures and results of dent induction. FIC was transplanted at an early stage of first cleavage, and then slits were made on the cortex on two sides of the transplanted site 3-5 min or 5-20 min after the FIC transplantation, in Holtfreter's saline (Hol.) and in a colchicine solution (Col.). Results in each case are shown on the right-hand. +, ± and – indicate the case of dent induction, weak reaction and no reaction, respectively.

DISCUSSION

The present report demonstrated that colchicine, a microtubule-depolymerizing agent, severely damaged the generation and growth of cleavage furrows in newt eggs, even when the agent was locally applied around the cleavage plane. Furthermore, the agent inhibited the induction of furrow-like dents by the FIC transplantation and furrow formation in isolated cortical fragments. These results were similar to those from a previous experiment in which microtubular poisons were injected into *Cynops* and *Xenopus* eggs (Sawai, 1992; Sawai and Yomota, 1992; Kubota and Sakamoto, 1993).

In the previous experiments when poison solutions were injected into cleaving eggs, we could not determine the range of the effect the poisons had on cleavage-furrow formation in operated eggs. The present experiment succeeded in roughly limiting the area on which colchicine acts as an inhibitor, and the results from experiments with cleaving eggs reveal that the microtubular poison directly interfered with the establishment of contractile structures in the cortex on the cleavage plane.

In a previous paper (Sawai, 1980), fragments of cortical layer with small entire cleavage furrows were isolated from *Cynops* eggs just after the onset of first cleavage, and were then grafted onto the animal hemisphere of uncleaved fertilized eggs. The fragments continued to divide on the host eggs and were separated into two parts. This result suggests that fragments with small furrows were ready for furrow formation on the presumptive cleavage plane in the fragments and for the contraction of the furrows to divide themselves into two parts. The present results showed that cleavage in cortical fragments with a furrow progressed in Holtfreter's ringer, as expected, but was completely inhibited in a colchicine solution. This means that colchicine injured directly the establishment and contraction of a furrow in a cortical fragment that had gained an ability to divide independently of the endoplasm or cytoplasmic structures.

We have shown by the FIC transplantation experiments (Sawai, 1972, 1983; Sawai *et al.*, 1969) that a furrow was established by an interaction of a cortical and a cytoplasmic factor . With regard to the relationship between the role of microtubule structures and that of the two factors in cortical events, several reports had indicated that microtubular poisons have no effect on the emergence of cortical changes such as travelling of a contractile wave, rounding-up of eggs

or increase of stiffness (Hara *et al*., 1980; Sakai and Kubota, 1981; Sakai and Shinagawa, 1983; Shinagawa, 1983; Yoneda *et al*., 1982), which are regarded as a reflection of a cortical change indispensable for furrow formation (Sawai, 1979; Sawai and Yoneda, 1974). From these findings, it seemed possible that the cortical factor could appear independently of microtubule structures. If this was true, furrow-like dents should form on the surface of eggs treated with microtubular poisons in response to induction by the FIC. However, the previous paper showed that this was not the case when the FIC was transplanted to the egg injected beforehand with the poisons. In the present experiment, the eggs were locally treated with the poison at a site where the FIC had been already transplanted. This resulted in the interruption of the induction of furrow-like dents, although the treatment was made before or after the emanation of induction.

The present results, taken together, confirm a previous suggestion that the microtubule structures are directly involved in the generation and growth of a cleavage furrow in newt eggs.

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