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A contractile ring-like mechanism in wound healing and soluble factors affecting structural stability in the cortex of *Xenopus* eggs and oocytes

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SUMMARY

Holes or tears have been made in the surface of unfertilized *Xenopus* eggs or oocytes in various buffered media and the surface response to the wound has been observed with the light microscope.

In the presence of calcium ion in the medium, a pigmented ring appears around the wound and constricts in purse-string fashion until the hole is closed. A highly pigmented scar remains. We show that the surrounding pigmented cortex is moved over the denuded cytoplasm when wound healing closes a large excision wound in the pigmented surface. This movement of surface is consistent with a model of a purse string, closing the hole by pulling existing surface over the denuded cytoplasm.

The presence of cytochalasin B in the medium inhibits wound healing completely. The sensitivity to the antibiotic is similar to that of the contractile ring in cytokinesis.

Wounds made in the surface of ripe ovarian oocytes do not heal in the presence of calcium ion. The healing mechanisms, or their sensitivity to calcium, thus appear during meiotic maturation or ovulation.

The egg cortex and membrane around wounds dissociate in the absence of calcium or in the presence of cytochalasin B. The cell cortex of oocytes also dissolves around wounds even in the presence of calcium. This cortical dissolution will not occur in the isolated cortex and thus requires soluble cytoplasmic factors. Models are proposed to explain these observations.

INTRODUCTION

The contractile properties inherent in the cortex of animal eggs have been shown to be involved in a variety of important activities in normal fertilization and development. (For recent reviews see Elinson, 1980; Vacquier, 1981.)

In addition, an induced cortical contraction was described by Holtfreter (1943) in his classical studies on the amphibian egg. He punctured the surface of the egg and was able to show that the wound would first gape open a bit and then close completely, even when large areas of the egg or embryo had been experimentally removed. During the wound closing movements a heavily pigmented ring formed around the hole and radial stress folds were often seen radiating out from

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the edge of the ring. Cortical pigment or surface dye marks were stretched towards the hole during closing. These observations suggested that a constricting ring around the hole was acting like a purse string in closing it, although he interpreted them as an 'active spreading' of a pigmented surface 'coat' (Holtfreter, 1943).

The papers of Holtfreter led to subsequent studies which have explored the mechanisms of wound healing in greater detail. Work with the electron microscope on the normal cortex, using thin sections (Bluemink, 1972; Gingell, 1970; Hebard & Herold, 1967; Luckenbill, 1971), has shown that two morphologically distinguishable cortical layers can be discerned. A thin 'felt-like' hypolemma of 0·1–1·0 μm thickness is found just under the plasmalemma and fills surface protrusions. A subjacent layer is 5–8 μm thick under the pigmented surface of the egg and contains pigment granules, cortical granules, membrane-enclosed vacuoles or cisternae and mitochondria. Most yolk is excluded from this dense, subjacent layer. These structures are diagrammatically summarized in Fig. 4A of this communication.

The cortical ultrastructure shows interesting changes during the healing of an experimentally produced wound. The thin hypolemma undergoes considerable thickening around the hole and fills numerous protrusions from the cell surface. Immediately underneath the thickened hypolemma, an extensive accumulation of membrane-enclosed vesicles can be seen. In the thicker, subjacent layer there is an accumulation of pigment granules, giving rise to the ring of heavy pigmentation which can be seen on the surface with the light microscope. Soon a circular ring of filaments develops around the hole (Bluemink, 1972; Luckenbill, 1971). It is 4–8 μm thick and is located between the intact, vacuolated cortex and the cytoplasmic exovate of the wound. It is formed either from, or adjacent to, the thickened hypolemma layer of the cortex. The filaments of the ring are roughly parallel and measure about 7 nm in diameter (Bluemink, 1972), a morphology similar to that seen in the contractile ring of the cleavage furrow (Schroeder, 1973) and consistent with the idea that they are actin microfilaments.

As the wound closes, the thickened hypolemma and ring of surface protrusions, the filamentous ring, the underlying accumulation of vacuoles and the deep ring of accumulated pigment all follow the edge of the wound as the hole becomes smaller and smaller in diameter. After closing, the final scar shows numerous residual surface protrusions and a substantial accumulation of pigment in the deeper subjacent cortex (Bluemink, 1972; Gingell, 1970; Luckenbill, 1971).

These fundamental observations have led to two major models to explain the events of wound closure. The first envisions an active spreading of the intact surrounding surface into the wound area (Holtfreter, 1943) or 'growth of new cell surface circumferential to the wound, allow(ing) the surface to expand' (Bluemink, 1972). The second lays emphasis on the movement of surrounding cortical pigment into the wound circumference, the formation there of a filamentous

ring, and its similarity to a contractile ring acting as a drawstring in closing the wound (Luckenbill, 1971).

In this paper we demonstrate that established pigmented surface is moved over the wound area. We present evidence that the contractile mechanism involved is inhibited by cytochalasin B (CB) in the medium. We show that the mechanism involved is either missing an active component(s) or is otherwise incapable of responding to a calcium trigger in full-grown ovarian oocytes. The responding mechanism thus seems to become active sometime during meiotic maturation or ovulation. Finally, we present evidence that the egg cell cortex is structurally destabilized around a wound when calcium is missing from the medium or CB is present in the medium. The oocyte cortex is similarly shown to become structurally destabilized around a wound even in the presence of calcium. Models are presented to explain these observations.

MATERIALS AND METHODS

Egg laying was induced in *Xenopus laevis* females by injections of 600–800 i.u. of human chorionic gonadotropin. Eggs were stripped from the cloaca, dejellied in 35 mM-beta mercaptoethanol at pH 8·9, washed and kept in 0·1 strength Ringer's solution. Ovarian oocytes were obtained by treating small pieces of whole ovary with collagenase (Sigma Chemical Co., St. Louis, Mo.) at 2 mg/ml in 0·1 M-phosphate buffer, pH 7·4 for 20 min at 35 °C (Schorderet-Slatkine & Drury, 1973). Freed oocytes were maintained in Amphibian Ringer's solution until use. Vitelline membranes and residual ovarian follicles were removed with forceps in 0·1 strength Ringer's (eggs) or 2·0 strength Ringer's (oocytes) before testing the cells.

Calcium-free medium contained 25 mM-Tris-HCl, pH 7·4; 80 mM-NaCl; 4 mM-$MgCl_2$; 2 mM-beta mercaptoethanol; 0·1 mM-phenylmethyl sulfonyl fluoride (protease inhibition); 0·02 % NaN_3.

Sometimes ethyleneglycol-bis (beta aminoethyl ether) N,N′-tetraacetic acid (EGTA) was added to calcium-free medium at 2 mM.

Cytochalasin B was purchased from Sigma Chemical Co., and was routinely tested for biological activity before each use by seeing whether it would inhibit cleavage furrow completion in fertilized eggs at a concentration of 10 micrograms per ml in the medium.

Photomicrography was done using a Leitz microscope and camera set-up with incident lighting.

The distribution of pigment around healed wounds on the surface of eggs was recorded by densitometry. A photomicrograph was taken of the surface and a positive transparency made by making a contact print onto Kodak Plus X film. A strip of positive transparency was cut out which included the scan line desired. This strip was positioned in a scanning attachment of a Gilford spectrophotometer and scanned, using a specially restricted exit 'slit' of approximately 0·5 mm diameter.

In all experiments 20–50 or more cells from several females were observed before pictures were taken or conclusions drawn.

RESULTS

Puncture wounds in either pigmented or unpigmented surfaces of the *Xenopus* egg will heal, but since events are more easily observed in the pigmented hemisphere, this surface was used. A puncture wound gapes open in the first 0·1–1·0 min. This fast retraction involves only the immediately peripheral egg surface. Then in the next 1·5–10 min a pigmented ring forms around the hole and it closes as previously described (Gingell, 1970; Holtfreter, 1943). Calcium ions in the medium are necessary and pH slightly lower than 7·0 is helpful in the healing process. In the range of calcium ions used in these studies, from 0·05–1·0 mM, the more the calcium the more vigorous the closing. The general event is shown in Fig. 1 where a large piece of pigmented cortex has been excized with fine forceps. In this cell, as in others that were tested, the healed wound shows a small, heavily pigmented scar when the cytoplasmic exovate has been removed (Fig. 1C). This represents the remnant of the pigmented ring which encircled the closing hole.

Radial stress folds can often be seen around the pigmented ring during the closing process. Furthermore, the healed surface shows little or no attenuation of the surface pigment over the area of the former wound, as can be seen in the photomicrographs and traces of Fig. 2. These observations would be most easily explained if the surrounding pigmented cortex was pulled, without much stretching and thinning, over the wound area.

A cortical drawstring would be a mechanism very similar to current concepts about the contractile ring which is involved in the deepening of the cleavage furrow in cytokinesis. The cleavage mechanism is known to be sensitive to cytochalasin B (Bluemink, 1971; de Laat *et al.* 1973; Luchtel *et al.* 1976; Selman *et al.* 1976). To see if wound healing also was inhibited by the antibiotic, we

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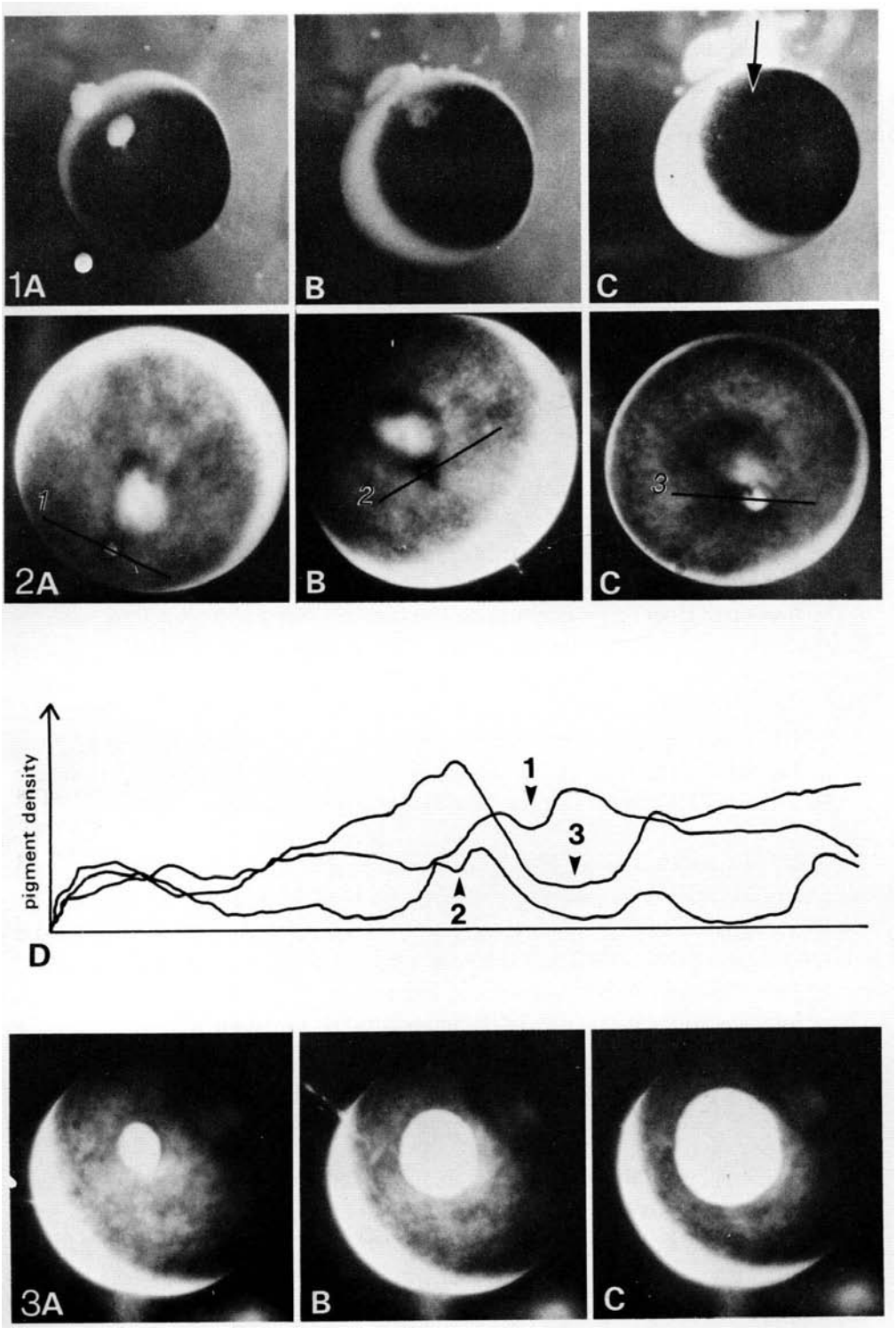
Fig. 1A, B, C. Photomicrographs of a single egg during the healing of a wound in the pigmented cortex. (A) A small piece of cortex was removed with forceps and the fresh wound has just undergone the initial gaping open. (B) Yolk is oozing from the wound during closure. (C) The wound has closed, leaving a pigmented scar (arrow). Note that the total pigmented area is reduced from the original area in (A). Mag. ×21.

Fig. 2A, B, C, D. Photomicrographs of three eggs showing the residual scars after wound healing. The trace lines go through the scar on each cell and approximately delineate the paths of the densitometric traces shown in 2D. The point in each scan which coincides with the centre of the scar is shown by an arrowhead. Note that there is little or no reduction of pigment around the scars, although the original wounds were of the order of size as that shown in Fig. 1A. Mag. ×33.

Fig. 3A, B, C. Photomicrographs of a single egg during the enlargement of a wound which occurs when the egg is in calcium-free or cytochalasin B-containing media. Note that there is a progressive dissolution of the cell surface at the wound edge. Mag. ×30.



Figs 1–3

added it to calcium-containing media at a concentration of 10 μg per ml. The result can be seen in Fig. 3. The initial gaping open of the slit occurred as usual but then the edge of the wound failed to stabilize and close. Instead, a slow and steady enlargement of the wound began, caused by the progressive dissolution of the pigmented cortex at the edge of the hole. This inhibition of wound healing occurred in all cells tested in both pigmented and unpigmented surfaces. We concluded that, like the contractile ring of cytokinesis, wound closure is inhibited by CB.

Previous workers have documented the requirement for calcium in the cortical contractions of wound healing. We removed calcium from the medium to see if endogenous calcium release could be detected in the wound healing process. The effect was always the same. The initial gaping open occurred as usual, but instead of ring formation and closure, a slow structural dissolution of the edge cortex began which slowly enlarged the hole. This dissolution occurred in both pigmented and unpigmented surfaces. It resembled the effect of CB in the presence of calcium but usually the dissolution process was slower than that caused by CB.

The progressive dissolution of the cortex at the edge of a wound, caused by CB or lack of calcium, always stopped after the cell had been substantially opened to the medium. If an egg was cut in half in calcium-free medium only a minimal dissolution occurred at the cut edge. These observations suggested that dissolution was an active process that required soluble cytoplasmic components. The concept was tested by opening a cell and immediately blowing away the cytoplasm with a stream of medium. An isolated cortex prepared in this way was stable for days in calcium-free medium. We concluded that cortical dissolution in the absence of calcium is a process which requires soluble components of the cytoplasm.

Finally, we decided to see if wounds would heal in ripe ovarian oocytes, or if meiotic maturation was also required for this cortical activity. To answer this question, large stage-5 and -6 ovarian oocytes were freed of their follicles and vitelline membranes and then punctured while in the same calcium-containing media that allowed vigorous wound healing in eggs. The answer was very clear. There was no initial gaping open of the wound, no ring formation around the hole and no closure at all in any cell tested. Instead, the hole slowly enlarged by dissolution of the cortex around the hole. The effect resembled closely the edge dissolution seen in the egg cortex when calcium was lacking or when CB was present.

We concluded that the cortical contractile mechanisms which close wounds in the surface of eggs in the presence of calcium ions are either missing functional components or are insensitive to a calcium trigger in oocytes. Wound-healing ability thus seems to arise during meiotic maturation or ovulation. We further noted that the ability of calcium to stabilize the cortical structure at the edge of wounds in eggs was absent in oocytes.

DISCUSSION

The observations reported here and those reported by others have led us to the conclusion that the closure of a wound in the cortex of the amphibian egg occurs as a consequence of the constriction around the hole of a ring of microfilaments. When a large area of cortex is experimentally removed from the pigmented surface of an egg, closure of the hole involves movement of the adjacent surface over the wound area. During the closing process radially arranged stress folds are often seen, originating on the edge of the contracting hole. Pigment and surface markers, adjacent to a wound, are pulled and elongated towards the central scar during closure (Holtfreter, 1943).

There seems to be relatively little stretching of the immediately adjacent pigmented surface during closure. This follows from our observation that there is little attenuation of cortical pigment around the scars of closed wounds. It follows also from the electron microscope studies of the healing process where it was shown that the cortical morphology, behind the closing pigmented ring and surface projections, is essentially identical to that of the undisturbed surface (Bluemink, 1972; Luckenbill, 1971). It does not have the appearance of newly formed surface.

Two observations support the idea that the constricting component of the pigmented ring around a wound contains actin microfilaments. The first is the morphology of the circumferential ring of microfilaments described by Luckenbill (1971) and especially Bluemink (1972), who measured their diameter at 6–7 nm. Both morphology and dimensions are consistent with the idea that they are composed of actin microfilaments. The other observation is our finding that CB inhibits wound healing, as it does cleavage furrow deepening (Bluemink, 1971; de Laat *et al.* 1973; Luchtel *et al.* 1976). It has been shown that CB inhibits actin filamentogenesis by blocking monomer addition at the growing ends (Brenner & Korn, 1979; Lin *et al.* 1980; MacLean & Pollard, 1980). It has been demonstrated also that structural actin exists in the cortex of *Xenopus* oocytes (Franke *et al.* 1976). Taken together, the evidence suggests that the constricting, pigmented ring around a closing wound includes a ring of actin microfilament bundles. If this is correct, its structural and functional similarity to the contractile ring of cytokinesis is obvious.

Since we believe that a constricting ring model of actin microfilaments is consistent with most or all existing data, we present a schematic model for wound closure which incorporates existing information into this mechanism. The model can be seen in Fig. 4.

The initial, fast gaping open of a wound (Fig. 4B) occurs most strongly and consistently in the pigmented cortex. It occurs in the complete absence of exogenous calcium and in the presence of CB. It involves only a very localized circumferential region of the cortex around the hole. For these reasons, we think that the initial, fast retraction of the wound edge corresponds to the initial

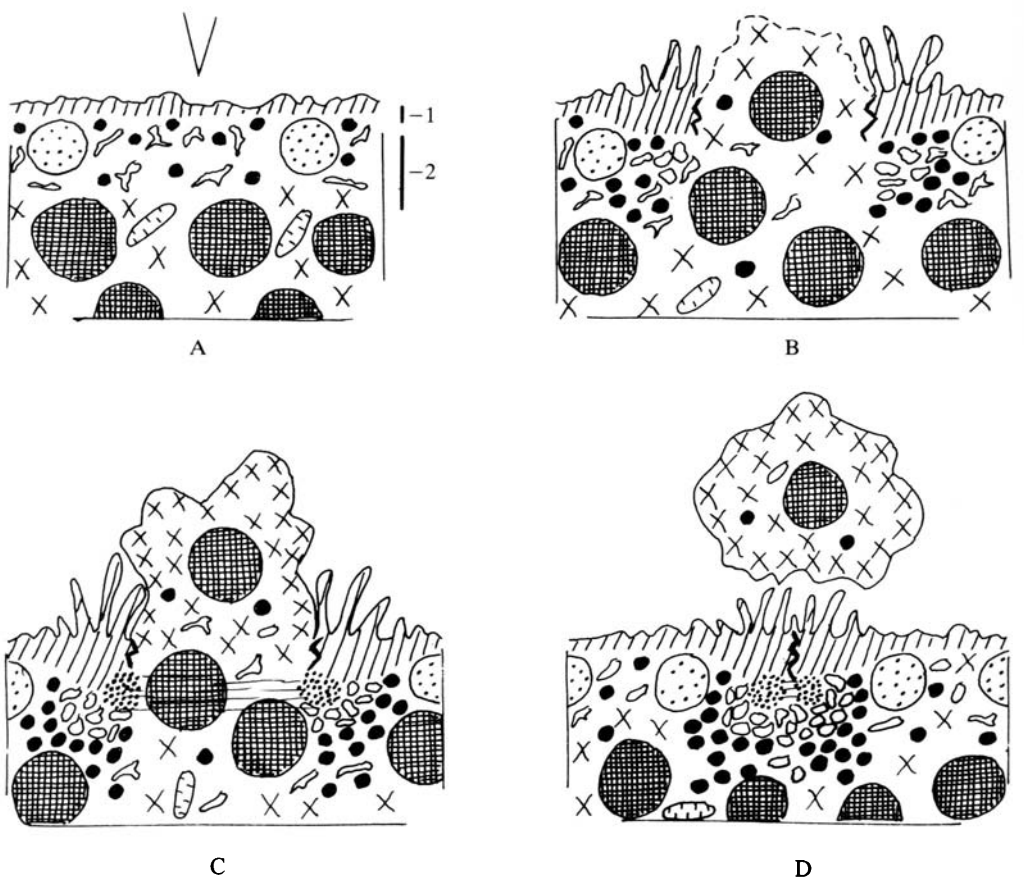


Fig. 4. Diagrammatic drawings to illustrate a model of a contractile ring-like mechanism that closes surface wounds in the *Xenopus* egg surface. For a discussion of the model see the accompanying text. (A) The normal, pigmented surface. Layer 1 is the outer, felt-like hypolemma. Layer 2 is the subjacent cortex, containing pigment, cortical granules and membranous cisternae. (B) A wound just after the initial gaping open response. Note the thickening of the hypolemma and the associated surface protrusions. (C) A microfilamentous ring has formed around the hole and the exovate has coagulated. (D) The microfilamentous ring and associated pigmented layer have closed into a scar. ● = pigment; ⊙ = cortical granules; = membranous reticulum; ⊕ = yolk; × = cytoplasmic matrix.

contractive thickenings of the outer hypolemma layer of the cortex and the subjacent pigment-containing layer. This contraction both starts the accumulation of pigment near the wound edge and thickens the hypolemma layer, causing extensive folding and formation of protrusions from the surface above (Bluemink, 1972; Luckenbill, 1971). Since cortical contractions are generally triggered by calcium ions (e.g. Gingell, 1970; Schroeder & Strickland, 1974), this fast retraction would presumably be due to endogenous calcium release at the edge of the wound caused by local trauma. It is not sensitive to CB and thus must not involve actin microfilamentogenesis (e.g. Brenner & Korn, 1979).

Secondarily, in the presence of exogenous calcium, the hole is stabilized by a CB-sensitive formation of microfilamentous actin bundles in, or close, to the outer hypolemma layer of the cortex and in a ring around the hole (Fig. 4C). The cytoplasmic exovate tends to coagulate to restrict the loss of endoplasm. Then, through a mechanism which is probably similar to the operation of the contractile ring of cleavage, the microfilamentous ring constricts to close the hole. In doing so it pulls adjacent cortex over the hole (Fig. 4D).

The dissolution of the cortical cytoskeleton and plasmalemma around the wound is an interesting phenomenon. It occurs in eggs in the absence of exogenous calcium or in the presence of CB. It occurs in oocytes even in the presence of exogenous calcium. It is an active process in the sense that it requires soluble cytoplasmic factors in order to occur.

A minimal model to explain these observations can be tentatively advanced. Both oocyte and egg cytoplasms would contain a soluble component which acts to take apart the cytoskeleton of the cortex. Its function is controlled by an inhibitor, which in the oocyte is removed by the raised calcium concentration at the edge of the wound due to exogenous calcium. During oocyte maturation the inhibitory cofactor is replaced by one which is removed when calcium levels fall to low values due to dilution of endogenous calcium at the edge of the wound when there is no exogenous calcium.

Since CB mimics the effect of low calcium in eggs and since CB is known to interfere in actin microfilamentogenesis, it is possible that actin microfilaments form the structural basis of the general cortical cytoskeleton. If this is so, then the hypothetical dissociation component could be a factor which modulates actin monomer/polymer interactions. It is obvious that other models to fit these data can be envisioned and that more work will be required to clarify this interesting system.

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