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Short Communication

Formation of the Amphibian Grey Crescent: Effects of Colchicine and Cytochalasin B

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Summary. The effects of colchicine and cytochalasin B on grey crescent formation in frog (*Rana pipiens*) and toad (*Bufo arenarum*) eggs were determined. Colchicine prevented the appearance of the grey crescent, but this inhibition was not due to the absence of an aster. Cytochalasin B did not inhibit grey crescent formation, nor did it inhibit certain activation events such as cortical granule breakdown or cortical contraction. Cytochalasin B caused a detachment of the cortex from the cytoplasm and induced the formation of a morphological grey crescent in non-activated eggs. The results suggest that microtubules may play several roles in grey crescent formation and that a change in the attachment of the cortex to the cytoplasm may also be involved.

Key words: Amphibian – Grey crescent – Colchicine – Cytochalasine B.

Introduction

The first indication of bilateral symmetry in the amphibian egg is the formation of the grey crescent. The grey crescent appears opposite to the site of sperm entry about one to two hours after fertilization. The sperm aster is probably responsible for this relationship since localized injection of sperm homogenate into eggs of the toad *Bufo arenarum* caused the appearance of an aster and the formation of the grey crescent opposite to the injection site (Manes and Barbieri, 1976, 1977).

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The mechanism of grey crescent formation is not known (see review by Brachet, 1977). Ancel and Vintemberger (1948) proposed that the egg cortex rotates relative to the cytoplasm to produce the grey crescent, and our recent observations support this idea (Elinson and Manes, 1978). Kubota (1967) felt that a local rigidity in the cortex overlying the aster could produce an asymmetry which led to the cortical rotation. On the other hand, Lovtrup (1965) argued that the grey crescent formed owing to an asymmetric contraction and not owing to a complete cortical rotation.

Since the sperm aster seems to be involved in grey crescent formation, we wanted to test the effect of colchicine which disrupts cytoplasmic microtubules including the aster. Also, since a contraction may be involved, we examined the effect of cytochalasin B (CB) which inhibits certain cellular contractions among other things (Wessells et al., 1971; Pollard and Weihing, 1974). In this paper, we report the effects of colchicine and CB on the morphological appearance of the grey crescent.

Materials and Methods

Rana pipiens and *Bufo arenarum* were used for these experiments. Procedures for obtaining gametes, inseminating, dejellying and activating eggs, and histology have been described in previous papers (Manes and Barbieri, 1976; Elinson, 1977; Elinson and Manes, 1978). Electrical activation of the eggs was performed by shocking them with a single pulse of 90 V and 100 msec duration. In experiments with *R. pipiens*, eggs were poisoned with colchicine either by injecting colchicine into the female or by microinjecting it into the egg (Elinson and Manes, 1978). CB was dissolved in DMSO at 1 mg/ml, or 3 mg/ml and diluted with phosphate-sucrose buffer (Fraser, 1971) for microinjection, or with 10% Ringer's solution for incubation. In experiments with *B. arenarum*, eggs were microinjected with sperm homogenate and scored for the appearance of the grey crescent opposite to the injection site (Manes and Barbieri, 1976, 1977). The homogenate was mixed before microinjection with colchicine, colcemid, or CB to test the effect of these drugs. In all experiments, eggs treated with DMSO in buffer or 10% Ringer's responded similarly to eggs in buffer or 10% Ringer's alone.

To score cortical granule breakdown, eggs were fixed and embedded according to Grey et al. (1974). Plastic was removed from thick sections, and the sections were stained with toluidine blue following the method of Kotani et al. (1973).

Results and Discussion

Effects of Colchicine

When *R. pipiens* eggs were poisoned with colchicine by injecting the female, the eggs were activated upon insemination but there was no sperm aster, no cleavage, and no grey crescent. Similarly, when activated eggs were injected with colchicine in contrentations above 0.4 mg/ml, no grey crescent appeared (Table 1). No grey crescent was obvious on eggs of *B. arenarum* injected with sperm homogenate plus colchicine or colcemid. In controls, the grey crescent formed opposite to the injection site (Table 2).

Table 1. Effect of colchicine and cytochalasin B (CB) on grey crescent formation. *R. pipiens* eggs were either activated by electrical shock or fertilized, and were then injected with 20–40 nl of solution. They were scored for grey crescent formation at 120 min

Injection solution	Number of eggs	Percent with grey crescent
(a) Activated eggs injected 10–20 min after activation		
Buffer	71	100
2 mg/ml colchicine	80	0
0.4 mg/ml colchicine	89	3.4
0.08 mg/ml colchicine	70	84
0.016 mg/ml colchicine	68	100
5% DMSO in buffer	53	100
50 µg/ml CB	73	100
10 µg/ml CB	69	99
2 µg/ml CB	73	100
(b) Fertilized eggs injected 50–60 min after insemination		
10 µg/ml CB	59	100

Table 2. Effect of colchicine and cytochalasin B (CB) on grey crescent position. *B. arenarum* eggs were microinjected with sperm homogenate mixed with test solution (total volume: 5–15 nl). The position of the grey crescent relative to the injection site was scored

Test solution	Number of eggs	Percentages with grey crescent opposite the injection site
Ringer's	36	96
4 mg/ml colchicine	12	0[a]
0.4 mg/ml colchicine	12	0[a]
0.2 mg/ml colchicine	12	75
0.37 mg/ml colcemid	23	8.7[a]
0.19 mg/ml colcemid	10	80
8, 4, or 2% DMSO in Ringer's	35	94
80 µg/ml CB	10	90
40 µg/ml CB	9	100
20 µg/ml CB	10	90

[a] Grey crescents were usually not obvious on these eggs

The inhibition of grey crescent formation by colchicine was not due to the destruction of the sperm aster. Activated, or activated and enucleated *R. pipiens* eggs in which we could find no aster formed grey crescents at the normal time. Although the aster is probably involved in the orientation of the grey crescent (Kubota, 1967; Manes and Barbieri, 1976, 1977), the aster is apparently not required for grey crescent formation.

These results imply that microtubules are required for grey crescent formation not only for asters but for another function as well. Whether microtubules are specifically involved in the movements to produce the grey crescent or whether they are needed to maintain normal cytoplasmic architecture as a condition for these movements should be determined.

Effects of Cytochalasin B

We were unable to inhibit grey crescent formation by either microinjecting or incubating eggs in CB. In the microinjection experiments, activated or fertilized *R. pipiens* eggs formed normal grey crescents when injected with CB (Table 1). (The injection of 20 nl of 10 μg/ml CB into cleaving eggs caused the regression of the furrow). Similarly, when CB was mixed with sperm homogenate and injected into *B. arenarum* eggs, the grey crescent formed opposite to the injection site as in control eggs (Table 2).

When dejellied, non-activated eggs were incubated in CB at 10 or 30 μg/ml in 10% Ringer's, CB altered the eggs in two ways. First, eggs kept in CB for about 2 h developed small white areas in the pigmented animal half. When the eggs were fixed and bisected, pigment granules appeared to be streaming from the cortical layer into the cytoplasm. Secondly, after incubation in CB, the relationship between the cortex and the cytoplasm changed. When unactivated eggs after removing the vitelline coat were pinched with watchmaker's forceps, the cortical layer seemed to be of the same consistency as the underlying cytoplasm, and it could not be grasped with forceps. When non-activated eggs treated with CB were pinched, the cortical layer receded over the cytoplasm from the cut, and it could be grasped with forceps and pulled away from the cytoplasm. CB therefore caused an alteration of the cortex or of the cortical-cytoplasmic attachments.

Although the above observations show that CB affected non-activated eggs, eggs incubated in CB for 2 h responded to electrical activation by breaking down of the cortical granules, raising a fertilization membrane and undergoing the cortical contraction (Elinson, 1975). The contraction looked different in CB-treated eggs as compared with control eggs, since it was more rapid and less pigment was moved towards the animal pole, leaving a grey band around the equator of the egg. Incubation of eggs for up to 8 h in CB at 10 μg/ml prior to activation did not inhibit the contraction. It is clear that CB affected the eggs; yet CB did not stop the cortical contraction associated with activation. In confirmation of the microinjection experiments, these CB-treated eggs usually formed a grey crescent at the normal time after activation. In some eggs, the presence of a grey crescent could not be assessed since the entire equatorial region was abnormally grey probably due to an incomplete relaxation of the cortical contraction.

Finally, CB could induce a morphological grey crescent formation in *non-activated* eggs. In this experiment, jellied eggs were placed in CB. Since the egg cell membrane of an non-activated egg is tightly apposed to the vitelline coat which is tightly held by the jelly, eggs landing in a tilted position remain so.

After about 6–7 h, tilted eggs had a grey crescent on the upper side. Upon electric shock, the eggs underwent activation including the cortical contraction and egg rotation. Untilted eggs in CB did not show a grey crescent, nor did tilted eggs in DMSO or 10% Ringer's. The induction of this grey crescent by CB required egg tilting but did not require egg activation. In the experiments described earlier to see whether CB could inhibit grey crescent formation, the grey crescent found was not induced to form by CB since it appeared on untilted eggs at the normal time and required egg activation.

When non-activated, colchicine-poisoned eggs were placed in CB, a very distinct morphological grey crescent formed on the tilted eggs.

We had hoped that CB would indicate whether a contraction was involved in grey crescent formation. This was not possible since when eggs were incubated in CB, CB failed to stop a known cortical contraction of the frog egg. Attempts to inhibit the cortical contraction by microinjecting CB into non-activated eggs were unsuccessful since wound healing did not occur at the injection site and this led rapidly to lysis of the egg. In non-activated eggs, however, CB-incubation caused a separation of the cortex and the cytoplasm and also induced the formation of a morphological grey crescent. We suggest that CB may be mimicking the events in normal grey crescent formation. The grey crescent probably forms owing to a rotation of the cortex relative to the cytoplasm (Ancel and Vintemberger, 1948; Elinson and Manes, 1978). One way to allow such a rotation is to detach the cortex from the cytoplasm. In CB-treated non-activated eggs, CB causes the separation of the cortex from the cytoplasm and gravity then causes the cytoplasm of tilted eggs to rotate relative to the cortex which is held in place by the vitelline coat and jelly. It would be interesting to know whether the CB-induced grey crescent is physiologically normal and whether a detachment of the cortex from the cytoplasm occurs prior to normal grey crescent formation.

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