Cytochalasin B II: Selective Inhibition of Cytokinesis in *Xenopus laevis* Eggs (35450)

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Carter (1) has described inhibition of cytoplasmic division (cytokinesis) with unimpaired nuclear division (karyokinesis) in mammalian cells treated with cytochalasin B (CB), a metabolite from the mold, *Helminthosporium dematioideum*. We report a similar effect of CB on the initial cleavages of *Xenopus laevis* embryos. Our data on both the duration and magnitude of drug effect define temporal limits for the act of cytokinesis and suggest that a pool of material which will be used for subsequent cleavages is available prior to the first cleavage. The demonstration that nuclear division can be separated from cytoplasmic division has been made before with both chemical and physical agents (2–7). However, because CB seems to have minimal effects on other cell processes (8), we felt that it would be an ideal tool for the definition of those events surrounding cytoplasmic division.

Methods. Batches of 10–15 eggs, fertilized within approximately 2 min of each other, were collected from *X. laevis* adults hormonally induced to mate (9). The outer jelly coats were removed using watchmakers forceps and the eggs were exposed to 1–10 μg/ml of CB (2.1×10^{-6} to 2.1×10^{-5} M) prepared by diluting, with distilled water, stock solutions of 1 mg/ml of CB in dimethyl sulfoxide (DMSO). Although CB is only sparingly soluble in pure water, it is readily soluble in dilute solutions of DMSO. Controls were exposed to 1% DMSO in distilled water, the maximum concentration of DMSO used in the experimental solutions. In our initial experiments we found that solutions of 10 μg/ml or more of CB caused pigment clumping and rotation of the eggs, both signifying death, while controls in 1% DMSO were unaffected. The remainder of our experiments were run with doses of 1–5 μg/ml. Further details of procedure, *ie.*, concentration of CB, time and duration of exposure to CB, are given in the text below and in the figure legend.

Results. We have focused our attention on the first two cleavages and the beginning of the third. CB produced consistent alterations in the external features of the cleavage furrow as well as in the internal structure seen in histological sections. The following external changes occurred when the eggs were placed in 5 μg/ml of CB immediately after removal of the outer jelly coat: (a) the first two cleavage furrows began at the usual time and position and with a normal appearance; the third cleavage furrow began at the usual time but was disoriented, *i.e.*, it was nearly meridional rather than equatorial; (b) the progression of the first two furrows around the egg appeared normal; (c) however, about the time each furrow was completed at the vegetal pole, "pigment separation" was observed in the depths of the furrow at the animal pole; (d) pigment separation became more pronounced and continued along the furrow which became shallower and finally disappeared leaving a white band of poorly pigmented cortex as the only surface indication of the cleavage plane. Events (c) and (d) are referred to below as "reversal" of the furrow.

Certain alterations in internal structure were seen in sections of fixed and plastic-embedded (10–12) eggs observed with the light microscope: (a) the nucleus had divided three times and the resulting eight nuclei appeared in correct position; (b) the cleavage planes were indicated in the animal

TABLE I. Degree of Cleavage Reversal Seen with Graded Doses of CB.

Dose (μg/ml)	Cleavage		
	1st	2nd	3rd
1	+[a]	++	Disorientation of plane
3	+++	++++	Disorientation of plane
5	+++++	+++++	Disorientation of plane

[a] Number of (+) indicates extent of pigment separation but not in strict proportionality, e.g., ++ greater than +, but not two times +. The drug was present at the indicated levels from shortly after fertilization until after the beginning of the third cleavage.

pole by a decreased density of yolk granules and a light accumulation of pigment granules; no suggestions of membrane dividing the egg into separate cells was seen along the former cleavage planes in contrast to separate cells seen in control eggs.

We found that the effects of CB were dependent on concentration within the range of 1–5 μg/ml (Table I). Solutions of 1 μg/ml and 3 μg/ml CB produced furrow reversal in the first two cleavages qualitatively similar to the effects described earlier for 5 μg/ml. However, the degree of pigment separation was less for the first cleavages at lower concentrations. This result suggests that a CB sensitive component(s) is present in a pool which is not replenished as this component is utilized during the next two cleavages.

As indicated in Table II, a 5 μg/ml solution of the drug produced reversal of the furrow if present at, or shortly after, the time the furrow was laid down in any particular region. However, when the drug was applied after the furrow was partially or fully completed, the completed portion remained unaffected; whereas the furrow produced subsequently showed the characteristic reversal. Note, for example, the eight eggs identified with a superscript *a* (Table II): the furrows in the animal half, completed prior to addition of the drug, were unaffected; whereas all the furrows in the vegetal half were reversed. In all cases reversal did not begin until the entire furrow was completed as far as the vegetal pole.

The full reversal effect on the first three cleavages could be produced by placing the embryos in a 5 μg/ml solution of the drug for 15 min during a fairly limited time prior to first cleavage: if the exposure to the drug ended earlier than 14 min before first cleavage, no effects were seen; however, when the 15-min exposure overlapped the 0–13 min preceding cleavage, the total effect on all three cleavages was seen in a high percentage of eggs (Fig. 1).

These latter experiments were not alone sufficient for determining the precise time of onset of CB sensitivity. A substantial time delay in washout of CB, for example, could mean that the onset was much nearer the initiation of the first cleavage. Therefore, we determined the minimum exposure time necessary for CB to produce furrow reversal. Fertilized eggs showing the first signs of the initial cleavage were divided into two groups. One group was placed in 1% DMSO control solution and the other was placed in the standard 5 μg/ml of CB solution. After 1-, 3-, 5-, or 10-min exposure to CB, a few of the eggs were removed and placed in 1% DMSO control solution for further observation. The resulting effects on cleavage in these eggs are noted in Table III. As indicated, about 50% of the eggs exposed to CB for only 1 min

TABLE II. Furrow Reversal Indicated by (+), No Effect by (—).

Time after beginning of 1st furrow (min)	Cleavage			No. affected/no. of eggs observed
	1st	2nd	3rd	
5	+	+	+	9/9
10	±[a]	+	+	8/8
15	—	+	+	6/6
20	—	+	+	5/5

[a] Animal pole furrow was unaffected while vegetal pole furrow was reversed. The eggs were placed in 5 μg/ml solutions of CB at the indicated times following the initiation of the first cleavage furrow.

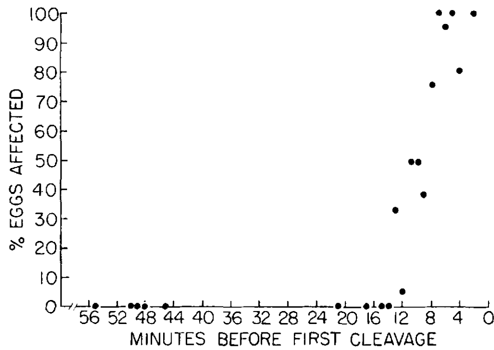

FIG. 1. Determination of the onset of sensitivity to CB: The eggs were placed in 5 μg/ml solutions of CB for 15-min periods after which the eggs were returned to control solutions containing 1% DMSO. The percentage of eggs showing furrow reversal is plotted against the time interval between the return of the eggs to control solutions and the initiation of the first cleavage furrow. Each point represents observations on 2–20 eggs.

showed complete reversal effects on the first three cleavages and 3-, 5-, and 10-min exposures affected a much higher fraction of eggs. Thus it appears that CB penetrates the eggs and produces its effect as early as 1 min after the beginning of exposure. If washout takes a similar length of time, these results, coupled with those of the previous experiment, indicate that the onset of CB sensitivity occurs between 10 and 13 min prior to the initiation of the first furrow.

Discussion. Our results suggest that cleavage, and perhaps cytokinesis in general, can be described as a three-step process involving: (i) nuclear division; (ii) initiation, progression, and completion of the furrow; and (iii) completion of the cell membranes which finally separate the daughter cells. CB apparently interferes with step 3 since nuclear division continues, and the furrows are initiated and completed normally (with the exception of the disorientation of the third furrow) before reversal is detected. These findings, as well as those from mammalian cell studies (1, 14), differ quite markedly from those of Schroeder (13) who found that CB arrests and reverses furrowing in sea urchin eggs within 1 min of drug application. His effects are correlated with a disappearance of cytoplasmic filaments in the furrow region. In Xenopus eggs, furrowing is completed normally before reversal begins. Similarly, mammalian cells constrict until fine cytoplasmic bridges remain between daughter cells; then reversal occurs producing binucleate cells (1, 14). We cannot explain the difference between Schroeder's observations and our own or those made on mammalian cells, nor do we have any evidence which indicates that filaments are directly affected in our system [but see (15–17)]. Our suggestion that CB affects certain membrane fusion processes is discussed below and elaborated further by Estensen (8).

TABLE III. Relative Numbers of Eggs Showing Cleavage Reversal After Brief Exposures to 5 μg/ml of Solutions of CB.

Exposure was begun when first furrow was initiated.

Duration of exposure (min)	No. affected/no. observed
1	8/15
3	12/13
5	9/11
10	6/7
15	2/2

An observation apparently unique to the *X. laevis* eggs is that CB exerts its full effect on at least three cleavages even though it is briefly present well before the time step 3 is normally carried out. Thus CB must interfere with some critical components(s) which is necessary for final separation of daughter cells and which is present prior to first cleavage.

A primary effect on macromolecular synthesis seems unlikely for the following reasons: (a) cleavage will still occur even when RNA and DNA synthesis is blocked (18–20); and (b) doses of CB which inhibit cytokinesis completely in mammalian cells, have little effect on protein synthesis or membrane synthesis (8). However, effects on ions or other small molecules or direct effects on macromolecules are not excluded.

Our data provide some important information about the target substance: (a) it is stored prior to first cleavage in amounts sufficient for cytoplasmic division in at least the first three cleavages. This conclusion is based on two findings. CB will affect the first

three cleavages although present just briefly prior to first cleavage; and, at lower doses of CB, there is a gradual increase in degree of furrow reversal in second and third cleavages. Substantial pools of other substances such as ribosomes and messenger RNA, exist in newly fertilized amphibian eggs (18). These substances, as well as the CB-affected substance, may be stored to compensate for the greatly reduced time available for synthetic activity following the initial cleavage in pregastrula embryos (21). Such storage may not be necessary for other dividing cells, *e.g.*, cultured mammalian cells, and this may account for the apparent reversibility of CB affect on other cells (1, 8).

(b) The total pool of substance apparently becomes available for CB-action rather abruptly 10–13 min before the first cleavage, and part of this pool becomes resistant to CB after the substance has been used in completing cytoplasmic division at each successive point along each cleavage furrow (as shown in Table II, this occurs about 10 min after the furrow has first appeared at a particular point). The time that a part of a furrow becomes resistant to CB may correspond to the completion of the daughter cell membranes at that place.

The time of the onset of CB sensitivity might be the time the substance is synthesized, the time the substance is released from a previously inaccessible area, *e.g.*, with the breakdown of the nuclear membrane in prophase, or the time the egg becomes permeable to the drug. Proteins necessary for cytokinesis in sand dollar embryos are synthesized rapidly within a brief time in early G_2 (4). If the action of comparable proteins in *X. laevis* embryos were blocked by CB, the sensitivity should appear during G_2. If CB affected some substance released upon disruption of the nuclear membrane, the sensitivity should appear in early prophase. No prediction can be made about the time one might expect a permeability increase to CB to occur. Comparing the data of Graham (22) to our results, we note that the actual time of appearance of CB sensitivity corresponds roughly to the end of G_2 and beginning of M phase. Thus our data do not yet permit us to distinguish between these alternatives.

(c) The final step in cytokinesis is the separation of the two daughter cells. This event must involve a process like membrane fusion, *i.e.*, the joining of two parts of the same cell membrane prior to the formation of two membranes. We believe this process depends critically upon the substance affected by CB. Further elaboration of the implications of this idea is given by one of us elsewhere (8).

Summary. In the presence of cytochalasin B (CB), fertilized *Xenopus laevis* eggs fail to complete cleavage although nuclear division and furrowing occur normally. The effects of varied dose levels and changes in timing of exposure to CB indicate that a pool of substance(s) needed for subsequent cleavages becomes available for CB action just before first cleavage.

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