Calyculin-A Induces Cleavage in a Random Plane in Unfertilized Sea Urchin Eggs

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Calyculin-A (CLA), a protein phosphatase inhibitor, has been known to induce cleavage resembling normal furrowing in unfertilized sea urchin eggs. In CLA-treated eggs, actin filaments and myosin assemble to form a contractile ring-like structure in the egg cortex; however, this occurs in the absence of a mitotic spindle or asters. Here, we investigated the relationship between the plane of CLA-induced cleavage and the intrinsic animal-vegetal polar axis in sea urchin eggs. The animal-vegetal axis was established using black ink to visualize the jelly canal located at the animal pole in the jelly coat surrounding the egg. We measured the acute angle between the jelly canal axis and the cleavage plane for both fertilized eggs and CLA-treated unfertilized eggs. Although the acute angle lay within 10 degrees for most of the fertilized eggs, it varied widely for CLA-treated unfertilized eggs. Measurements of the diameter of blastomeres revealed that cleavage of fertilized eggs took place in the mid-plane of the egg, but that CLA-induced divisions were unequal. These results suggest that neither the orientation nor the location of the CLA-induced cleavage furrow is related to the animal-vegetal polar axis of the egg, even though the furrowing mechanism itself is not dissimilar to that in fertilized eggs.

Oocytes, or unfertilized eggs, of various animal species have polarities that determine the architecture of embryos. Sea urchin eggs are spherical, and microscopic examination suggests that the cytoplasm is generally distributed symmetrically. However, it is known that the animal-vegetal polar axis is already present in unfertilized sea urchin eggs. These eggs are surrounded by a jelly coat that is penetrated radi-

ally by a single channel, called the jelly canal. The cell cortex that lies underneath the jelly canal is the animal pole of the egg (1, 2). In fertilized eggs the first and second cleavages occur in planes that contain the animal-vegetal axis of the egg, while the third cleavage occurs in a plane perpendicular to the axis. The cleavages take place by constriction of the contractile ring (CR) formed in the cortex at the division site. However, the molecular basis for these cleavage patterns has not been clearly elucidated.

Calyculin-A (CLA), a potent inhibitor of protein phosphatases 1 and 2A (3, 4), has been known to induce a cleavage-like morphological change in unfertilized sea urchin eggs (5). It first induces polymerization of actin in the cortical layer of the eggs. The polymerized actin is then bundled to form a CR-like structure that encircles the cell. The ring then contracts to cleave the cell into two parts. There are some differences between this event and normal cleavage: the CR-like structure does not disintegrate during or after contraction in CLA-treated cells, whereas in normally dividing cells the mass of the CR is substantially reduced (6). Furthermore, the CR-like structure is not induced by microtubule structures as in normal cleavage, where the position of the CR is determined by the mitotic apparatus (7).

The exact mechanism of formation of the CR-like structure induced by CLA is not well understood. However, phosphorylation of some proteins that function in this process has been considered (8). Myosin regulatory light chain has been shown to be phosphorylated during this process, and such phosphorylation would cause contractility of the actin-myosin structure in the cortex (9).

Since the furrowing induced by CLA is similar in many respects to normal cleavage during cell division, it would be interesting to know how the plane of the CLA-induced

furrowing is related to the animal-vegetal axis of the egg. Is it possible that the plane is determined by the intrinsic polarity of the unfertilized egg?

Two species of sea urchins, *Lytechinus variegatus* (Lamarck) and *Lytechinus pictus* (Verrill), were used for this study. Adults were purchased from Gulf Specimen Marine Laboratories of Panacea, Florida, and were maintained in a seawater aquarium in our facility at the Marine Biological Laboratory, Woods Hole (MA).

Mature eggs were obtained by intracoelomic injection of 10 mmol l^{-1} acetylcholine chloride into the body cavity. Black ink (Bokuju; Kuretake Co., Japan) was dialyzed against seawater for more than 6 h. Immediately after they were shed, the *L. variegatus* eggs were suspended in black ink/seawater mixture (1:4) for about 1 min. After the eggs were washed gently with seawater several times, they were fertilized or exposed to 10 μmol l^{-1} CLA (a generous gift from Drs. Nobuhiro Fusetani and Shigeki Matsunaga) dissolved in seawater at room temperature (23–25 °C).

The microdrop method (10) was used to culture the eggs in a drop (about 10 μl) of seawater. The drop, separated by air within a seawater-moistened ring of filter paper, was sealed with Valap between coverslip and glass slide. Using this method, we were able to make extended observations of the eggs, which have a thick jelly coat, without deforming them. The fertilized eggs cleaved in about 60 min after fertilization, while the CLA-treated unfertilized eggs cleaved 30–60 min after initiation of the treatment. When *L. pictus* eggs were used, unfertilized eggs were exposed to 30 μmol l^{-1} CLA.

Light microscopic observations were carried out on a Leica DMRA microscope equipped with a Plan Fluotar 40×/0.70 N.A. objective lens, using differential interference contrast optics to visualize details. A Hamamatsu cooled CCD camera (c5985) was used to capture the images, which were recorded on a DVD recorder (Panasonic DMR-E50). Images were transferred from the DVD to an image analysis computer. Using Meta Imaging Series software (ver. 7.0 r3, from MDS Analytical Technologies, Downingtown, PA), the acute angle between the jelly canal and the cleavage plane, and the diameters of two halves of divided eggs were measured. *F*-tests were used to find significance in the difference of the measured values.

Using black ink as a staining probe, previous studies have demonstrated that the jelly canal in the thick jelly coat on sea urchin eggs marks the position of the animal pole (2, 11). Figure 1 illustrates *L. variegatus* eggs stained with black ink after gentle washings with seawater. Particles of ink remaining in the jelly canal formed a black mark.

For fertilized eggs, the acute angle measured between the first cleavage plane and the axis of the jelly canal lay within 0 to 20 degrees in 84% of the eggs (Figs. 1D and 2, gray bars). In contrast, for CLA-treated unfertilized eggs, the acute angles were scattered between 0 and 90 degrees (Figs. 1F, H, and 2, black bars). We confirmed that the acute
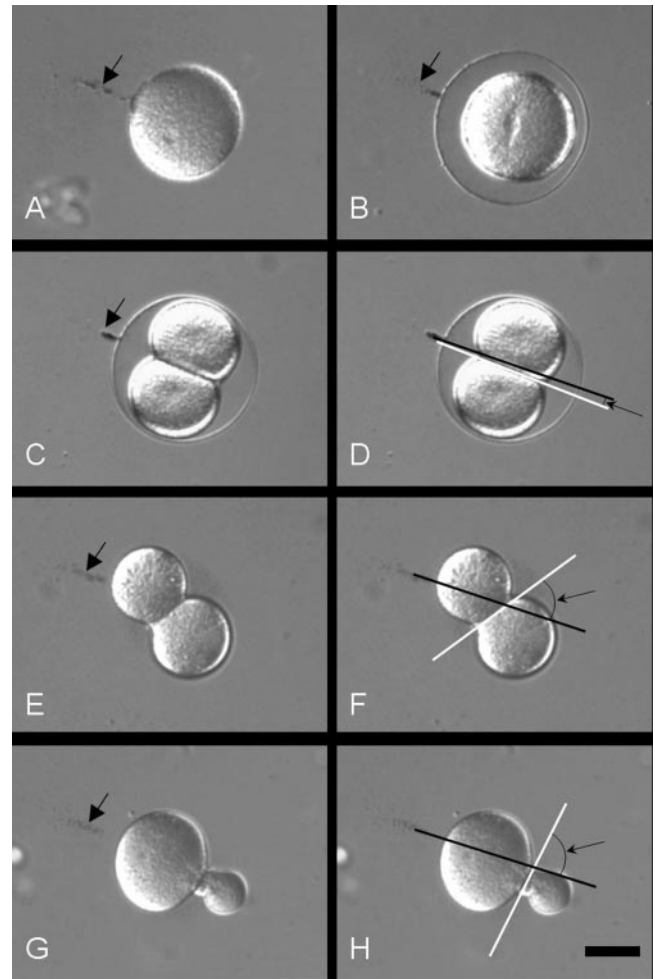

Figure 1. Light microscopic observations of *Lytechinus variegatus* eggs stained with black ink. (A) Unfertilized egg. (B) Fertilized egg at the streak stage. (C, D) Embryo at 2-cell stage. (E, F) Unfertilized eggs treated with CLA undergoing equal cleavage. (G, H) Treated eggs just after unequal cleavage. Black particles of ink remained within the jelly canal after washings (arrow in A), fertilization (arrows in B and C), or treatment with CLA (arrows in E, G). In D, F, and H, the white and black lines indicate the cleavage plane and jelly canal axis, respectively, and the thin arrows point to the acute angles between them. The cleavage plane is almost parallel to the axis defined by the jelly canal in the fertilized egg as shown in D, but is not so in the CLA-treated unfertilized eggs seen in F and H. Bar = 5 μm.

angles for the fertilized eggs and the CLA-treated unfertilized eggs were significantly different ($P < 0.05$ by *F*-test).

We also noticed that the size of the two blastomeres formed by CLA-induced division was often unequal. In our previous study (5) we reported that the CLA-induced cleavages take place at planes near the mid-plane in many sea urchin species. However, we had not carried out a quantitative analysis. Therefore, in the current study we used the following calculations to estimate the location of the CR-like structure that would have given rise to the blastomeres of different sizes.

Assume the initial unfertilized egg to be a perfect sphere. Then slice the model sphere into two parts at an arbitrary
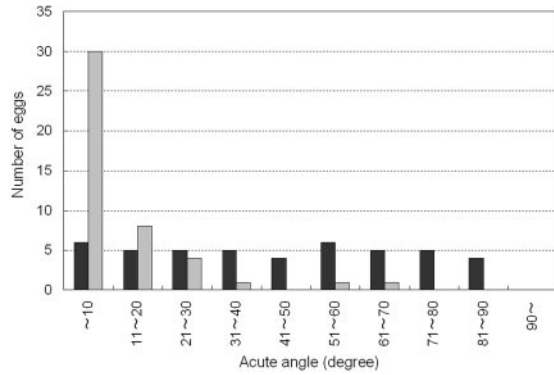
Figure 2. Acute angles between the axis of the jelly canal and the cleavage plane *(Lytechinus variegatus)*. The number of fertilized eggs (gray bar, n =45) or CLA-treated unfertilized eggs (black bar, n = 45) is plotted *versus* the acute angle.

plane (Appendix Fig. 1). The volumes of the fragments arising from division through this plane can be calculated, giving rise to the volume percentages of these fragments (as fractions of the original sphere; Appendix Table 1).

In turn, the approximate volumes of the two blastomeres actually formed by CLA-induced division are calculated from their diameters, yielding their relative volumes. Comparison of their volume percentages with those calculated for the model yields the estimated location of the division plane.

As detailed in the Appendix, we chose to slice half of the model sphere into 10 equally thick sections. Then we calculated the volume percentages of the blastomeres that would have resulted if the cleavage plane had fallen at each sectional plane (Appendix Fig. 2 and Table 1). Finally, the number of actual eggs whose calculated cleavage planes fell into each range was plotted against the distance of the cleavage plane from the mid-plane.

As plotted in Figure 3, the cleavage planes of fertilized eggs were concentrated around the mid-plane of the eggs (gray bars). In contrast, the division planes of CLA-treated *L. variegatus* and *L. pictus* unfertilized eggs were scattered between the midsection of the egg and the distal cortex (black bars).

These observations and analyses reveal that the orientation and location of the CLA-induced cleavage furrow bear no relation to the animal-vegetal polar axis of an unfertilized egg. Furthermore, the orientation and location of the CLA-induced cleavage furrow are independent (data not shown). Rather, it is apparent that every cortical part on the unfertilized egg can form a furrow upon induction by CLA.

In the normal first and second cleavages of fertilized sea urchin eggs, the location of the cleavage furrow is determined in two steps. First, the mitotic apparatus is formed such that its longitudinal axis at metaphase is perpendicular to the animal-vegetal axis. Second, the cleavage furrow is formed in the cortical layer where astral microtubules emanating from the two centrosomes merge with each other, as shown by various micromanipulation experiments (7). The role of astral microtubules in determining the cleavage

plane has also been shown by removal of various microtubular regions from mitotic sea urchin eggs (12). Therefore, in fertilized eggs the direct determinants of cleavage position are likely to be the astral microtubules. Furthermore, related micromanipulation experiments demonstrate that the cleavage plane can be altered from that containing the animal-vegetal axis (13–15).

As described above, the plane of cleavage induced by CLA bears no relationship to the intrinsic animal-vegetal axis of the sea urchin egg. Furthermore, estimated locations of the division plane revealed that there was no preference for the plane to pass through the center of the egg.

In CLA-treated unfertilized eggs, microtubules are found in the cytoplasm, but they do not converge on specific sites. Rather they seem to be randomly distributed in the cytoplasm (5). Also, griseofulvin, a strong inhibitor of microtubule polymerization, did not interfere with the induction of the cleavage-like morphological change (5). Therefore, the change is unlikely to be induced by polarized microtubule structures.

Then a question arises regarding CLA-induction of a CR-like structure: how is the plane of its formation determined in each egg? We reported earlier that the structure is formed through fusion of a random array of cortical F-actin bundles into a ring (5, 8). Myosin or some F-actin-bundling protein activated by phosphorylation may play an important role in the
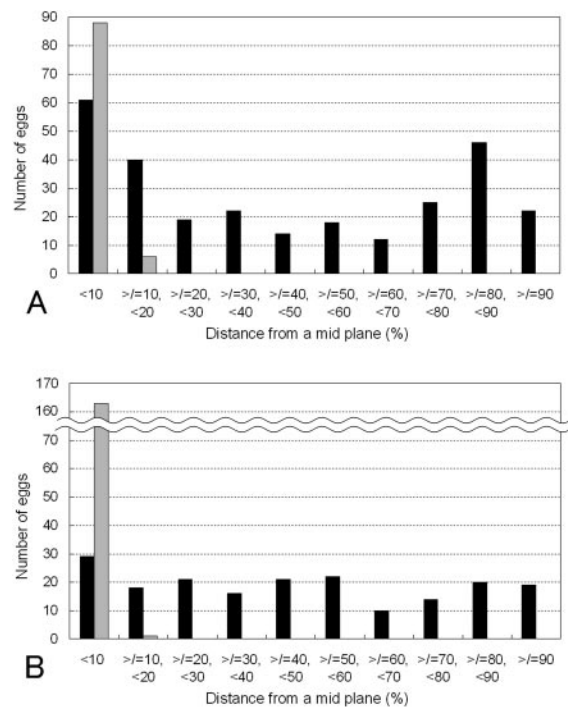

Figure 3. Distance between the division plane and a mid-plane that contains the center of the egg (A: *Lytechinus variegatus*, B: *L. pictus*). The radius of a model egg was dissected into 10 equal segments. The number of fertilized eggs (gray bar, n = 94 (A) and 164 (B)) or CLA-treated unfertilized eggs (black bar, n = 279 (A) and 190 (B)) is plotted for each segment in which the division plane (as estimated using the calculations detailed in the Appendix) is contained.

F-actin bundle formation and subsequent fusion of bundles. It could be that the structure is formed in a stochastic manner by spontaneous fusion of cortical F-actin bundles.

Although the location of the CR during cleavage in fertilized eggs is determined by the distribution of mitotic microtubules, the CR giving rise to a polar lobe during cleavage of certain molluscan eggs does not depend on microtubules (16). It would be interesting to inquire whether some other mechanism related to CLA-induced furrowing localizes the CR that gives rise to the polar lobe.

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Appendix

Method of estimating the location of the initial cleavage furrow from the relative sizes of the blastomeres.

When a perfect sphere is cut by a plane at an arbitrary location, we obtain a large and a small fragment (Appendix Fig. 1A). The volumes of these fragments are calculated by the following equations:
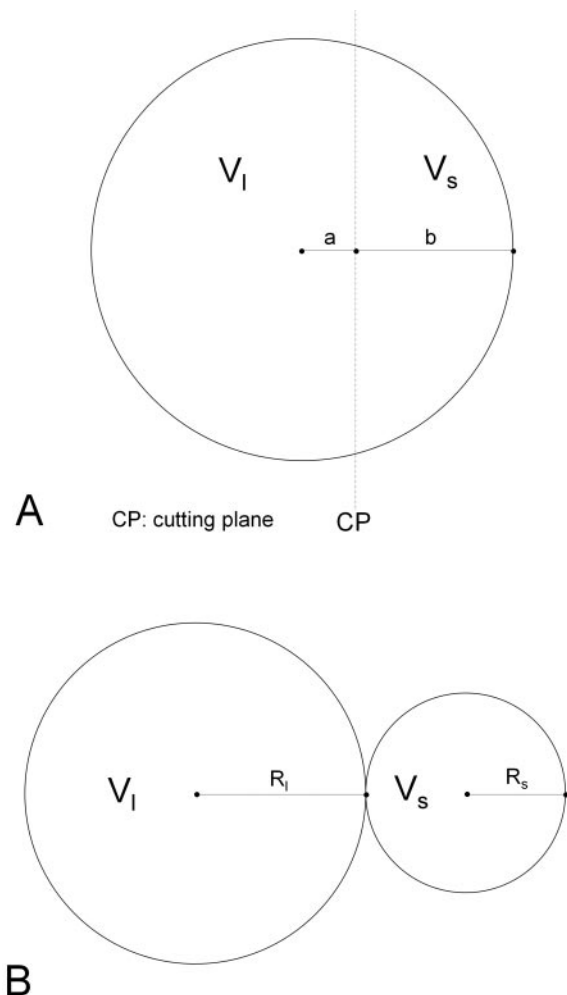
Appendix Figure 1. (A) A perfect sphere cut into unequal fragments. (B) Model of blastomeres arising from cleavage in (A).

Appendix Table 1

Relationships used to estimate location of the initial cleavage furrow

Distance between center of sphere and sectional plane (% of radius of sphere)	Volume of large fragment (ratio %)	Radius of large fragment (Rl)*	Volume of small fragment (ratio %)	Radius of small fragment (Rs)*
90	4.15842 (99.27%)	0.99758	0.03037 (0.73%)	0.19354
80	4.07150 (97.20%)	0.99058	0.11729 (2.80%)	0.30366
70	3.93432 (93.92%)	0.97933	0.25447 (6.08%)	0.39311
60	3.75315 (89.60%)	0.96406	0.43563 (10.40%)	0.47027
50	3.53429 (84.37%)	0.94494	0.65450 (15.63%)	0.53861
40	3.28401 (78.40%)	0.92209	0.90478 (21.60%)	0.60000
30	3.00860 (71.82%)	0.89555	1.18019 (28.18%)	0.65557
20	2.71433 (64.80%)	0.86535	1.47445 (35.20%)	0.70607
10	2.40751 (57.47%)	0.83143	1.78128 (42.53%)	0.75199
0	2.09440 (50.00%)	0.79370	2.09440 (50.00%)	0.79370

* Radius of undivided egg is taken as 1.

Volume of large fragment: $Vl = (2/3)\pi r^3 + \pi(ar^2 - a^3/3)$,

and

Volume of small fragment: $Vs = (2/3)\pi r^3 - \pi(ar^2 - a^3/3)$,

where "r" is the radius of the original sphere (= a + b in Appendix Fig. 1A) and "a" is the distance from the center of the original sphere to the cutting plane (CP).

Appendix Table 1 shows the relationship between the volumes of these fragments and the distance from the center of the sphere to each cutting plane (with half of the sphere sliced into 10 equally thick sections; Appendix Fig. 2). Columns 3 and 5 of the table show the radii of spheres (Rl and Rs, Appendix Fig. 1B) that have volumes equal to the larger and smaller fragments.

In turn, the volumes of the two blastomeres arising from cleavage of the eggs are calculated from their radii. The initial location of the contractile ring that gave rise to those blastomeres is then estimated by comparing the volume ratios of the observed blastomeres with the volume ratios of the model blastomeres listed in column 2 or 4 of the table.
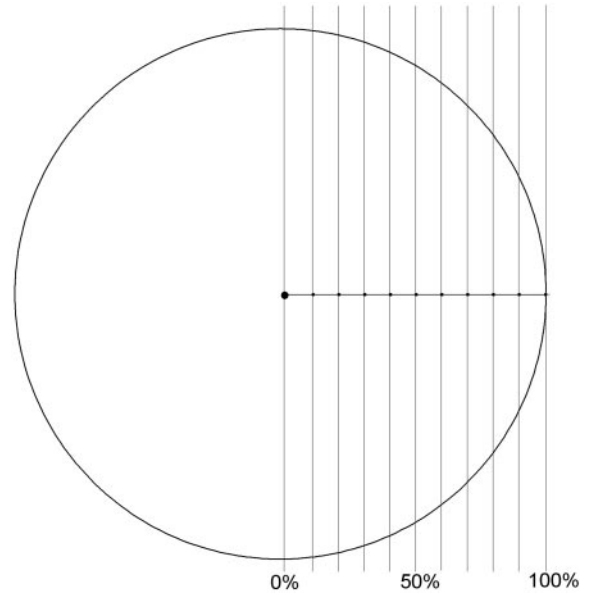
Appendix Figure 2. (A) The model sphere in (A), with half of the sphere sliced into 10 equally thick sections.