was 3.5-fold higher than in the absence of the hormone. Assay of lipolysis was carried out for 3 hours in the absence of the hormone, and for 3 hours at 37°C in 5-ml vials containing 3×10^5 cells, 0.4 μ*M* isoproterenol, and increasing concentrations of the test serum. Portions of the medium were then taken, bovine serum albumin was removed by trichloroacetic acid precipitation, and the glycerol content was determined by the triglyceride C-37 Rapid-Stat test (see *Pierce Catalog*, 1976). The amount of glycerol released was 12 and 140 nmole per 3×10^5 cells per 3 hours in the absence and the presence of 0.4 μ*M* isoproterenol, respectively. Insulin at 10 ng/ml inhibited 87 percent of the glycerol released. The titer of antibodies to insulin was determined by a solid phase radioimmunoassay (*27*). Half-maximum activity was obtained at 1.0 μl and 1.5 μl of the test serum per milliliter for glucose oxidation and inhibition of lipolysis, respectively.
13. The fraction eluted from the protein A–Sepharose column was pure IgG as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and mercaptoethanol performed as described by U. K. Laemmli [*Nature (London)* **227**, 680 (1970)]. Only the heavy and light chains of IgG were detectable on the gel. No bands that corresponded to insulin were seen.
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Calcium Ionophore Polarizes Ooplasmic Segregation in Ascidian Eggs

Abstract. *Calcium ionophore A23187 promotes ooplasmic segregation and orange crescent formation in eggs of the ascidian* Boltenia villosa. *When eggs were exposed to a gradient of A23187 the orange crescent was induced to form in the region corresponding to the highest concentration of ionophore. This result is consistent with the hypothesis that a local increase in intracellular calcium polarizes cytoplasmic localization in the ascidian embryo.*

Cell fate during early embryogenesis is thought to be determined by the localization and segregation of cytoplasmic components (*1*). The distribution of these components in many embryos is established by an extensive episode of cytoplasmic rearrangement, known as ooplasmic segregation, which is initiated shortly after fertilization. Although ooplasmic segregation is often one of the earliest visible indications of the future embryonic axis, little is known about how the various cytoplasmic components are directed to specific regions of the egg. It was recently proposed that ooplasmic segregation may be organized by an intracellular gradient of free calcium (*2*). This proposal is supported by experiments with developing fucoid eggs which suggest that rhizoids are extended toward areas of high calcium concentration (*3*, *4*). Transient elevations in intracellular calcium may also play an important role in the activation and early development of animal eggs (*5*). The present study provides evidence that the segregation of cytoplasmic regions with specific morphogenetic fates and the subsequent expression of bilateral symmetry in ascidian eggs are directed by a local increase in intracellular calcium at the time of fertilization.

We investigated the role of calcium in ooplasmic segregation by treating *Boltenia villosa* eggs with the divalent ionophore A23187 (*6*). *Boltenia villosa* eggs were selected because, after fertilization, they exhibit a pronounced segregation of pigmented cytoplasmic regions which is readily followed by light microscopy. Ooplasmic segregation is initiated by the streaming of cortical cytoplasm, composed of orange pigment granules and associated mitochondria, into the vegetal hemisphere of the egg, where it collects around the point of sperm entry as an orange crescent. A clear cytoplasm, derived from the germinal vesicle, flows into the vegetal hemisphere in the wake of the orange cytoplasm. The clear cytoplasm temporarily forms another crescent immediately inside the orange crescent and eventually returns to the animal hemisphere with the male pronucleus. A cytoplasmic lobe is also protruded from the vegetal region of the egg at the time of maximal crescent development. The plane of the first cleavage furrow bisects the orange crescent, dividing the zygote into right and left halves characteristic of the bilaterally symmetric ascidian embryo. The developmental fate of the orange crescent cytoplasm of *B. villosa* eggs is identical to that of the yellow crescent cytoplasm of *Styela partita* eggs, the subject of Conklin's classical observations on ooplasmic segregation (*7*). Orange crescent cytoplasm is specifically partitioned to the precursors of the tail muscle and mesenchyme cell lineages in tadpole larvae.

Ionophore A23187 was previously shown to activate ascidian eggs and promote several partial cleavages (*8*) but, since the species investigated lacked eggs with pigmented cytoplasmic regions, no information on the ooplasmic segregation was obtained. In order to obtain this information, eggs were dissected from the gonads, washed with artificial seawater (ASW), and placed in petri dishes containing A23187 dissolved in ASW at several different concentrations (*9*). When the eggs were examined 20 to 30 minutes later a large proportion had developed orange crescents. The highest proportion of eggs with orange crescents (65 to 80 percent) was obtained with 4 μ*M* A23187. Less than 3 percent of eggs incubated in ASW lacking A23187 developed orange crescents. Orange crescent formation did not depend on the concentration of external calcium, sodium, or hydrogen ions since it was not affected when eggs were treated with A23187 in calcium-free ASW containing 3 m*M* EGTA, in ASW in which choline was substituted for sodium, or in

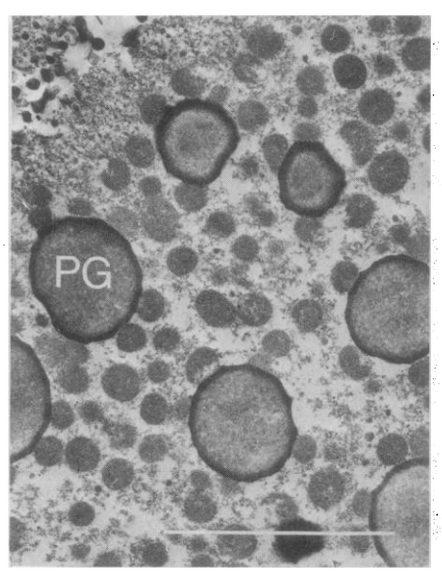

Fig. 1. Electron micrograph of the orange crescent region of an A23187-activated egg. Pigment granules (*PG*) with associated mitochondria are present. Scale bar, 10 μm.

ASW ranging in *p*H from 5.5 to 8.5.

The sequence of events during ooplasmic segregation was similar in fertilized and A23187-activated eggs. Orange crescents were first observed 5 to 10 minutes after either insemination or the addition of A23187. A cytoplasmic lobe was formed in the vegetal region of activated eggs at the stage of maximal crescent formation, and a crescent of clear cytoplasm was usually seen beneath the orange crescent at this time. As in fertilized eggs, the clear cytoplasm of activated eggs returned to the animal hemisphere. In some of the activated eggs an orange crescent was formed but a crescent of clear cytoplasm did not develop in its wake. All the eggs that behaved in this way were immature, as judged by the presence of a germinal vesicle. Thus, breakdown of the germinal vesicle and resumption of the maturation divisions is not a prerequisite for orange crescent formation.

The orange crescent of *B. villosa* eggs, like the mesodermal crescent of other ascidian eggs (*10*), is primarily an aggregation of mitochondria and pigment granules. Since the assay for ooplasmic segregation relies on orange coloration, it is possible that A23187 activates the migration of the pigment granules but not the mitochondria. In order to test this possibility, A23187-activated eggs were examined by electron microscopy. As shown in Fig. 1, the orange crescent region of these eggs contained mitochondria as well as pigment granules. The results indicate that A23187 induces the migration of the two major orange crescent organelles as in normal ooplasmic segregation.

Although these results show that A23187 causes ooplasmic segregation in activated eggs, they do not elucidate the relation between local concentrations of calcium and egg polarity. The orange crescent may be focused on a portion of the egg surface which is exposed to the highest or to the lowest concentration of A23187. It is also possible that orange crescent polarity is unrelated to differences in the A23187 concentration but instead is directed by a determinant of polarity in the egg. Hence, eggs were exposed to an A23187 gradient (*4*) to probe the relation between the site of orange crescent formation and the local calcium concentration.

Small glass fibers (100 to 200 μm in diameter) were soaked in A23187 stock solution (*9*), air-dried, and cemented onto polylysine-treated glass cover slips. The cover slips were placed in small petri dishes containing 3 ml of ASW. A steep gradient of ionophore is set up around the glass fibers under these conditions (*4*). The eggs were pipetted into the petri dishes, allowed to settle near the fibers, and manually aligned so that one side contacted the fiber or was very near to it. When eggs positioned in this fashion were examined 20 to 30 minutes later, most of those that were activated formed orange crescents on the side nearest the fiber (Fig. 2A). Eggs located more than about 200 μm from the fiber remained inactivated. The distance of each orange crescent's midpoint from the point of the egg nearest the fiber was estimated in degrees around the circumference of the egg. It was found that 82 percent of the activated eggs formed orange crescents with midpoints 45° or

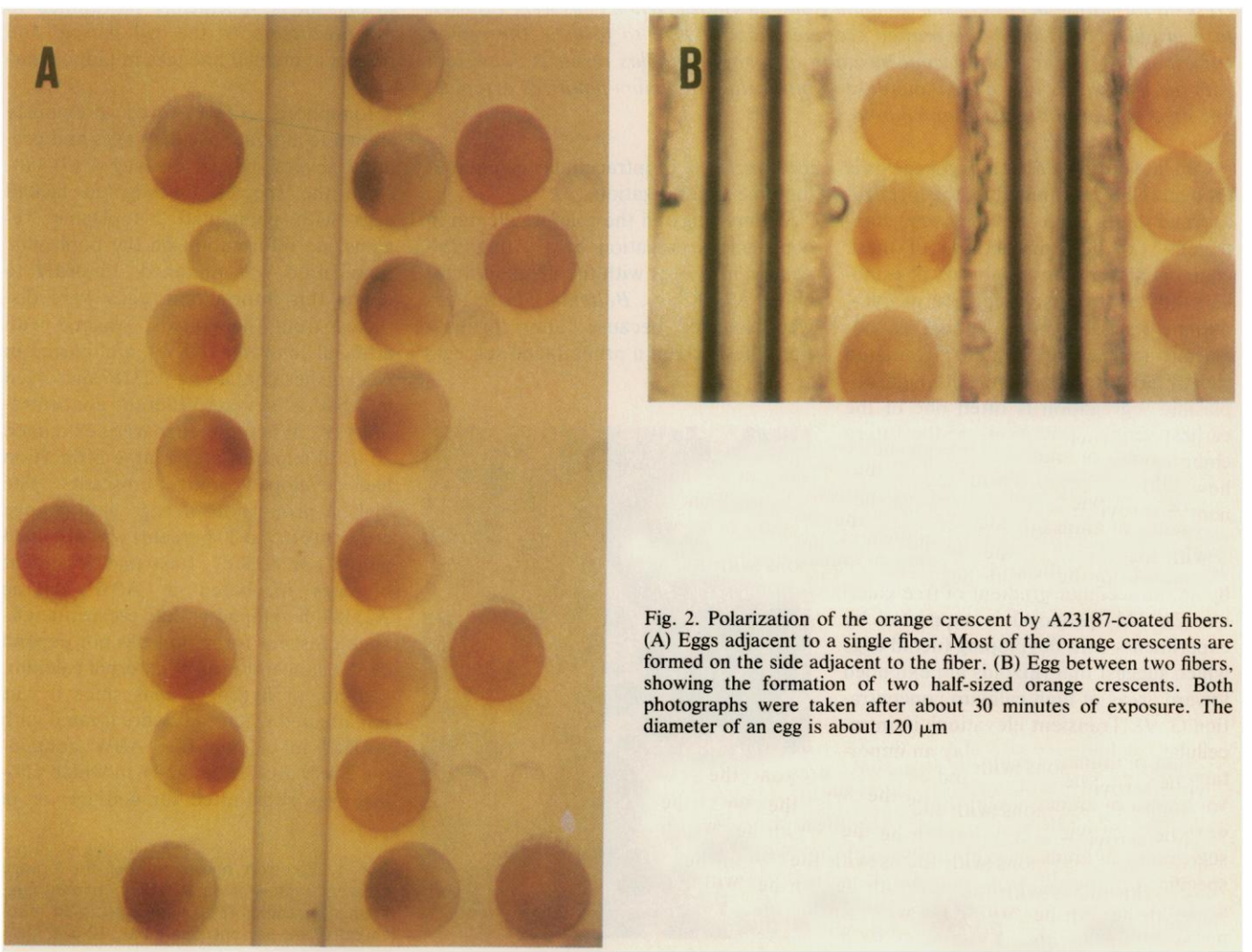

Fig. 2. Polarization of the orange crescent by A23187-coated fibers. (A) Eggs adjacent to a single fiber. Most of the orange crescents are formed on the side adjacent to the fiber. (B) Egg between two fibers, showing the formation of two half-sized orange crescents. Both photographs were taken after about 30 minutes of exposure. The diameter of an egg is about 120 μm

less from the point nearest the fiber. Fourteen percent of the activated eggs formed orange crescents with midpoints between 45° and 135° from the point nearest the fiber, while only 4 percent formed crescents on the opposite side of the egg (between 135° and 225° from the point nearest the fiber). The clear crescent and cytoplasmic lobe were also focused on the side of the egg nearest the fiber. When transient cleavage furrows were formed they were extended along a plane perpendicular to the fiber and bisected the orange crescent. These results cannot be explained by a tendency of the fiber itself to polarize ooplasmic segregation, since the orange crescent appeared at random positions along the circumference of A23187-exposed eggs aligned adjacent to untreated glass fibers.

The polarization of ooplasmic segregation toward high concentrations of A23187 suggests that the egg perimeter is totipotent for the formation of an orange crescent. If this is correct, two orange crescents might be expected to develop in eggs that contact two A23187-coated fibers simultaneously. To test this possibility, two A23187-coated fibers of approximately equal diameter were cemented 100 to 150 μm apart on a cover slip. Eggs were pipetted into petri dishes containing these cover slips, allowed to settle, and positioned adjacent to the two fibers and between them. Eggs positioned between the two fibers often contacted both of them at opposite poles. About 1/2 hour after alignment most of the activated eggs adjacent to the outer margins of the fibers formed an orange crescent facing the fiber. While most of the eggs positioned between the fibers formed a single orange crescent centered between the points of contact, a few (10 to 15 percent) formed two orange crescents, each about half the normal size and centered at the points where the eggs contacted the fibers (Fig. 2B). These results indicate that multiple foci of ooplasmic segregation can exist in a single egg and support the hypothesis that there is no predetermined polarity of cytoplasmic localization in the ascidian egg.

In summary, it appears that a gradient of A23187 is able to polarize ooplasmic segregation and the establishment of bilateral symmetry in ascidian eggs. To our knowledge this is the first report of polarity determination by A23187 in an animal egg, although it was previously shown that A23187 polarizes plant cells (*3, 4, 11*). Since A23187 appears to activate eggs by releasing calcium from intracellular storage depots (*5*), the simplest interpretation of our results is that local elevations in calcium initiate and polarize ooplasmic segregation in the ascidian egg. This process, which may involve activation of a cytochalasin-sensitive cortical microfilament system (*12*), is probably triggered by sperm penetration in inseminated eggs. These experiments suggest that local changes in intracellular calcium may be a key factor in organizing the distribution of cytoplasmic determinants and the subsequent pattern of early embryogenesis.

WILLIAM R. JEFFERY*
Marine Biological Laboratory, Woods Hole, Massachusetts 02543, and *Department of Zoology, University of Texas, Austin 78712*

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* Send correspondence to permanent address at the University of Texas.

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