"CLEAVAGE" AND CORTICAL GRANULE BREAKDOWN IN RANA PIPiens OOCYTES
INDUCED BY DIRECT MICROINJECTION OF CALCIUM

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ABSTRACT
Microinjection of ~0.3 μg of calcium into maturing oocytes of Rana pipiens after nuclear dissolution resulted in cleavage-like constrictions, cortical granule breakdown, and formation of a structure resembling a two-cell embryo. Mg²⁺, Na⁺, or K⁺ did not induce any of these reactions. Larger amounts of Ca²⁺-induced contraction over the entire surface of oocytes or eggs, but did not induce cleavage-like constrictions; smaller amounts of Ca²⁺ produced either a local cortical granule reaction or the formation of one large and one small “blastomere.” Furrow formation was not observed during normally induced maturation until after germinal vesicle breakdown. The location of microinjected Ca²⁺ determined the orientation of the resulting furrow. Ca²⁺-induced cortical granule breakdown occurred in full-grown nonmaturing oocytes near the site of injection. Cortical granule breakdown also occurred in maturing oocytes (after germinal vesicle breakdown but before second meiotic metaphase), but only in the blastomere containing the injected Ca²⁺. As expected, in mature oocytes (at second meiotic metaphase) cortical granule breakdown occurred over the entire oocyte surface, including both blastomeres. The results indicate that furrow formation and cleavage-like constrictions may be directly influenced by Ca²⁺, and that functional contractile elements are present near all areas of the oocyte surface. Furthermore, Ca²⁺ injection initiates localized cortical granule breakdown in full-grown immature and maturing oocytes.

Amphibian oocytes grow for months, or even years, while arrested in prophase of the first meiotic division. When full-grown, they mature in response to certain steroid hormones by reinitiating meiosis (13, 20, 23). About halfway through the approx. 36 h required for maturation in Rana pipiens, the large oocyte nucleus, or germinal vesicle (GV), ruptures. Since the GV sequesters at least some materials made during oogenesis (5, 6, 11, 22), its dissolution allows for new macromolecular interactions. Maturation ends when meiosis is again arrested, this time at second meiotic metaphase. The cells, now termed eggs, are capable of being activated either by pricking or
by fertilization. Subsequent to activation, which is often scored by observing cortical granule breakdown, eggs begin to divide and embryogenesis proceeds. Profound biological changes occur during the transition from oocytes (nondividing cells) to eggs (cells ready to undergo rapid division). Knowledge about the disposition and control of cellular mechanisms during maturation is thus a critical prerequisite to an overall understanding of the important changes that occur during this phase of oogenesis. Recent evidence suggests that divalent ions in particular play a crucial role in these processes.

Calcium ions have been implicated, at least in oocytes and eggs, as a determinant for furrow formation and wound contraction. Injection of sufficient calcium chelator ethyleneglycolbis-(β-amino ethyl ether)N,N′-tetraacetic acid (EGTA) blocks cleavage of the Xenopus laevis egg (1). Wound healing and polycation-induced contractions are not seen in Ca2+-free media unless large amounts of Sr2+ or Ba2+ (but not Mg2+) are substituted (10). Calcium has also been directly tied to contraction by experiments showing a local contractile response at the site where Ca2+ was ionophoretically injected (10; and J. F. Ash, as cited in Wessells et al. [27]).

Although clearly less direct, the divalent ionophore A-23187 provides an additional means for investigating Ca2+-mediated processes. It has been used to release Ca2+ from mitochondria (15) and sarcoplasmic reticulum (18), and to trigger at least some of the responses characteristic of activation reactions in eggs from several species (24, 25). When combined with other treatments, ionophore A-23187 induced parthenogenetic development of sea urchin eggs (4). Ionophore, and by implication free calcium, activates mature amphibian eggs, causing cortical granule breakdown and cortical contractions, although cleavage or parthenogenetic development has not been reported for any amphibian egg. Ionophore induction of activation responses in R. pipiens oocytes, however, appears to be related to the stage of oocyte maturation at the time of treatment because it causes vitelline membrane elevation and cortical granule breakdown beginning 1–2 h after GV dissolution (2). Thus, since cortical granules obviously have the capacity to respond precociously to ionophore, as opposed to fertilization or a mechanical stimulus, the remaining 16–17 h of maturation must partially involve the organization or development of the capacity to propagate this cortical response (2).

In preliminary experiments during which R. pipiens oocytes (after GV breakdown) and eggs were exposed to ionophore A-23187, we observed surface changes characteristic of cortical granule rupture, surface contractions, and furrowing suggestive of cleavage. To directly evaluate the role of Ca2+ in such responses, we microinjected Ca2+ into amphibian oocytes and eggs. The results presented here illustrate that direct injection of calcium induces surface contraction, cortical granule breakdown, and, in certain cases, furrow formation and even cleavage-like constrictions in both maturing oocytes and activable eggs.

MATERIALS AND METHODS

Commercially obtained (Hazen, Alburg, Vt.) Rana pipiens were kept at 4°C until pithed before ovariectomy. Ovaries were maintained in amphibian Ringer’s solution as were individual oocytes that had been manually dissected from their investing follicular epithelium with watchmakers’ forceps. Maturation was initiated, when desired, by adding 1 µg/ml desoxycorticosterone (DOCA) to the Ringer’s solution. Meiosis proceeded to second metaphase within 32–40 h at 18°C, at which time the oocyte could be mechanically activated with a fine glass needle. Oocytes were enucleated by puncturing the animal pole with a blunt glass needle and gently squeezing the sides of the oocyte with watchmakers’ forceps, until the GV emerged. After enucleation, oocytes were transferred from Ringer’s to Steinberg’s solutions for 30–60 min to promote healing.

Injections were performed with a single micropipette, prepared by the method of Smith and Ecker (22), that injected 17.3 ± 0.3 nl, which increased the volume of the oocytes less than 1%. CaCl2, MgCl2, KCl, and NaCl were dissolved in glass-distilled water at concentrations such that the desired weight of cation would be contained in 17.3 nl. Weights, rather than concentrations, of injected cations are given because at least some ions become rapidly compartmentalized and are not freely available; thus, we have no information on cellular or effective concentrations. Solutions were, unless specified, injected equatorially about 0.3–0.4 mm deep while the oocytes or eggs were retained in holes punched in a thin 2% agar layer on the bottom of a petri dish. The precise location of the injectate was determined by dissecting fixed oocytes or eggs and relating the location of a small yolk-free area (representing the injectate) to the orientation of surface and other morphological features. Oocytes or eggs were fixed for 2–3 h at 4°C with 3% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4). Samples were processed and Epon embedded by standard methods (8) before they were sectioned and exam-
RESULTS

After 24–26 h exposure to DOCA, oocytes were injected with 17.3 nl of Ca\(^{2+}\) (0.001–3.0 \(\mu\)g), Na\(^+\) (0.01–1.0 \(\mu\)g), K\(^+\) (0.01–1.0 \(\mu\)g), or Mg\(^{2+}\) (0.01–1.5 \(\mu\)g). Cleavage-like constrictions occurred when 0.3 \(\mu\)g of Ca\(^{2+}\) was injected, although larger doses destroyed some (at 1 \(\mu\)g) or all (at 3 \(\mu\)g) of the oocytes. No effect appeared after Na\(^+\) injections except that oocytes receiving 1.0 \(\mu\)g cytolized. Oocytes became swollen and turgid after receiving 0.1 \(\mu\)g or more of K\(^+\) but were activable 10 h after injection by pricking, as were control DOCA-treated oocytes. Low doses of Mg\(^{2+}\) produced no noticeable effect, although 0.1–1.5 \(\mu\)g caused the cells to swell, become turgid, turn grayish-white, and appear cytolized.

Calcium injections produced four different responses,\(^1\) only two of which resemble cleavage (Fig. 1a). Cleavage-like responses varied in relation to the dose of Ca\(^{2+}\) and, depending upon the external appearance of the oocyte or egg, are referred to as "normal" or "partial" constriction (Table I). About 2–5 min after injection of 0.3 \(\mu\)g of Ca\(^{2+}\), the first sign of normal constriction appeared. A small depression formed about 90° away from the injection site in eggs or maturing oocytes whose GV's had broken down. This linear depression deepened, spread around the oocyte, and within 15–20 min appeared to have separated the egg into two parts. Some sectioned oocytes, although not all, appeared completely bisected.

Partial constriction followed microinjection of 0.1 \(\mu\)g of Ca\(^{2+}\) and was similar to the normal constriction (obtained with 0.3 \(\mu\)g of Ca\(^{2+}\)) except that the resulting portions of the egg were unequal in size (Fig. 1a). The injected portion included about one quarter of the oocyte's or egg's diameter and approx. 5% of its volume. Normal or partial constrictions occurred only after GV breakdown (Table I). Each started within 5 min of injection and exhibited the characteristically convoluted surfaces only on the Ca\(^{2+}\)-induced section of the oocyte or egg.

Microinjection of Ca\(^{2+}\) also induced two surface responses not involving cleavage-like reactions. The affected surfaces, in both cases, resembled the surface reactions seen on the Ca\(^{2+}\)-injected side of the egg and were similar to contractile responses of surfaces bordering healing wounds (3, 10). One response is called "complete surface reaction" (CSR). When large doses of Ca\(^{2+}\) (\(\geq 1\) \(\mu\)g) were injected into full-grown oocytes, maturing oocytes, or eggs, their entire surface became rough-looking, ragged, and convoluted (Fig. 1c). The second response, "partial surface reaction" (PSR), involved a limited portion of the oocyte's surface and occurred when smaller doses of Ca\(^{2+}\) (\(\leq 0.3\) \(\mu\)g) were injected into full-grown or maturing oocytes (before GV breakdown). The amount of Ca\(^{2+}\) injected after GV breakdown determined which cleavage-like response occurred.

Cleavage Response During Maturation

As previously implied, calcium injections did not induce constrictions in full-grown oocytes not stimulated by hormones. To evaluate the effect of maturational state, oocytes were injected with Ca\(^{2+}\) at various times during maturation. The results showed that the capacity to constrict developed during maturation and was invariably present after GV breakdown. Mixing GV contents with oocyte cytoplasm presents at least one obvious mechanism for activating new processes. To test this question, we injected Ca\(^{2+}\) into oocytes enucleated before hormone exposure. Before or at various times after initiation of maturation, both enucleated and control maturing oocytes received Ca\(^{2+}\). All oocytes were incubated in Ringer's for appropriate time-periods before exposing them to DOCA. Thus, all oocytes received Ca\(^{2+}\) about 40–42 h after removal from their follicles. The results (Table III) are clear. Neither the presence nor absence of the GV had any effect on whether or not Ca\(^{2+}\)-induced constriction occurred.

Orientation of Cleavage Furrows

Preliminary results showed constriction occurring on various axes. Therefore, we systematically

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\(^1\) In this report, the term cleavage (and other, related terms) is used to represent a deep constriction of the surface of the oocyte that may or may not completely bisect it. While this Ca\(^{2+}\)-induced reaction has several features that superficially resemble those of the first cell division after fertilization, the differences between these two processes may be more informative; i.e., while Ca\(^{2+}\) may be acting directly on the contractile systems in both cases, its injection perhaps bypasses the normal factors or processes derived from the subcortical cytoplasm effecting the controlled release of the cation (see references 12, 16, 17).
injected maturing oocytes with 0.3 μg of Ca²⁺ at the animal pole, equator, or vegetal pole to test whether or not the site of injection determines the plane of constriction. The plane was equatorial for both animal- and vegetal-pole injections but was longitudinal after equatorial injections (Fig. 1). The results thus show that the site of the injectate determined the plane of constriction. Also, oo-

Figure 1 (a) Cleavage in response to injected Ca²⁺. (I) The various responses (see description in text) to injected Ca²⁺ are diagrammatically illustrated. (II) The difference in cleavage response after Ca²⁺ injection into various locations in the oocyte is shown schematically. X marks the site of injection; T = time (in min) after injection; CSR = complete surface reaction; PSR = partial surface reaction. (b) Oocytes were obtained and exposed to DOCA for 25 h before injection with Ca²⁺. A section 1-μm thick, stained with toluidine blue, shows the extent of cleavage reaction. (c) Higher magnification of section shown in (b). Note how the injected blastomere has a fuzzy appearance due to convolutions over the entire surface. (d) Photograph of whole fixed oocyte is shown to illustrate external features of cleavage furrows.
cytes injected closer to the vegetal pole responded somewhat faster than those injected near the animal pole.

**Cortical Granule Breakdown**

Cortical granules ruptured after Ca$^{2+}$ injection. There were no cortical granules present under areas of the cell exhibiting a surface reaction in oocytes examined after receiving Ca$^{2+}$, nor were they present in the injected portion of partially or normally constricted cells. However, cortical granules remained intact in areas of oocytes that showed no response to injected Ca$^{2+}$. As expected, since eggs undergo a complete cortical response when punctured with the micropipette, cortical granules in fully mature eggs exhibited cortical granule breakdown after injection.

**TABLE I**

*Amount of Ca$^{2+}$ Required to Induce Cleavage-like Constrictions*

| No. oocytes | Dose  | CSR | Normal | Partial |
|-------------|-------|-----|--------|---------|
| 5           | 3     | 0   | 0      | 0       |
| 20          | 1     | 6   | 0      | 0       |
| 10          | 0.5   | 10  | 0      | 0       |
| 22          | 0.3   | 0   | 20     | 0       |
| 20          | 0.1   | 0   | 1      | 10      |
| 20          | 0.03  | 0   | 0      | 0       |
| 20          | 0.01  | 0   | 0      | 0       |
| 15          | 0.001 | 0   | 0      | 0       |

* Oocytes from four different frogs were isolated and exposed to desoxycorticosterone. The following day, after germinal vesicle breakdown, various amounts of Ca$^{2+}$ were injected.

† Responses: CSR = complete surface reaction; normal and partial responses are described in the text.

**TABLE II**

*Maturation State and Appearance of Cleavage-like Response*

| Time injected after DOCA | No. oocytes | Germinal vesicle | Response |
|--------------------------|-------------|-----------------|----------|
| h                        |             |                 |          |
| 0                        | 5           | +               | 0 0 0 5  |
| 2                        | 10          | +               | 0 0 10  |
| 6                        | 5           | +               | 0 0 5   |
| 22                       | 14          | -               | 6 0 0   |
| 24                       | 20          | -               | 18 0 0  |
| 30                       | 5           | -               | 0 0 0   |
| 36                       | 5           | -               | 5 0 0   |
| 44                       | 10          | -               | 7 3 1   |

* Oocytes from four different female frogs were removed and exposed to DOCA at time zero. At indicated times after exposure, 0.3 µg of Ca$^{2+}$ was injected.

† Plus sign (+) indicates intact vesicle; minus sign (-) indicates vesicle has broken down.

§ Responses: normal and partial responses are described in text; PSR = partial surface reaction (see text).

**DISCUSSION**

Calcium has previously been correlated with contractile responses in oocytes, eggs, and embryos (1, 2, 3, 10, 14, 19, 25; and J. F. Ash, as cited in Wessells et al. [27]), but, to the best of our knowledge, this is the first report of a Ca$^{2+}$-induced contractile response even superficially resembling cleavage. The results suggest a model for Ca$^{2+}$-induced constriction or cleavage that can be developed with two primary assumptions. These are that Ca$^{2+}$ diffuses away from the injectate and that some specific, or critical, concentration of Ca$^{2+}$ activates some contractile elements. We have assumed that these elements are microfilaments, but other Ca$^{2+}$-triggered systems with similar properties could be invoked without altering the essence of our argument. We envisage Ca$^{2+}$ as diffusing from the injectate toward the surface. When a critical concentration of the ion develops at or near the surface closest to the injectate, contraction begins generating the "surface response" described above. As the ions continue to diffuse, the area exhibiting this reaction grows until, at some point, the Ca$^{2+}$ concentration falls below the critical level needed to induce contraction. This point represents the limit of the surface reaction and
delineates the line where furrowing or cleavage starts, at least in oocytes or eggs otherwise capable of undergoing this response. Cortical granule breakdown also occurs in the Ca\(^{2+}\)-affected area, although in mature eggs granule breakdown spreads by normal means over the entire surface.

We assume that Ca\(^{2+}\) diffuses away from the injection site, but we do not imply that all the injected ion remains free in the cytoplasm. It is well known, for example, that mitochondria sequester Ca\(^{2+}\). Recent evidence indicates that they may also play a physiological role in Ca\(^{2+}\) regulation (7). Since mitochondria appear swollen in the injected side of oocytes and eggs (our unpublished observation), they may, in fact, be taking up at least some in the injected Ca\(^{2+}\).

Cleavage-like furrows appeared only where the area undergoing a contractile response bordered normal surface. Once initiated, these furrows tended to deepen. It remains to be determined why furrowing continues for several minutes until the oocyte or egg is deeply grooved or, in some cases, even bisected.

Major differences exist between Ca\(^{2+}\)-induced normal constriction and the first cleavage after fertilization. Ca\(^{2+}\)-induced planes occurred in any orientation (see below), while cleavage subsequent to fertilization had a defined orientation. Also, the surfaces of the two "blastomeres" formed by Ca\(^{2+}\) injection differed from each other (Fig. 1a-d), i.e., the injected side of the oocyte or egg was highly convoluted and appeared similar to surface morphology derived from contractile responses near healing wounds (3, 10). Constriction also started faster in response to Ca\(^{2+}\) injections (2-5 min) than in response to fertilization (2 h). Finally, Ca\(^{2+}\) generated a response at any time after GV breakdown (see below) while cleavage due to fertilization occurred only after the completion of maturation. Ca\(^{2+}\)-induced constriction varied slightly from one experiment to another, in that some groups of oocytes responded more quickly or completely.

Oocytes are first capable of constricting in response to injected Ca\(^{2+}\) after the time of GV breakdown, although nuclear contents are not required, as demonstrated by the enucleation experiments. At least some indications of contractile responses are apparent in oocytes not treated with hormones, i.e., complete or partial surface reactions. Thus, we are unable to say that the ability to contract appears de novo during the course of maturation. An alternative possibility is that, before the time of GV breakdown, cleavage is mechanically restrained due to the tight attachment of the oolemma to the vitelline membrane. These two structures begin separating near the time the GV breaks down (21).

Ca\(^{2+}\)-induced constriction first occurs after GV breakdown, a minimum of 16 h before fertilization-induced cleavage is possible, and can occur anywhere on the oocyte surface. Thus, mechanisms controlling cleavage after fertilization are apparently not functioning at the level of de novo organization of localized contractile systems. Since there is some indication that contractile properties exist throughout the oocyte even before fertilization, another possibility is the development of an activating or triggering system (see references 9, 12, 16, 17).

Cortical granules break down only on the Ca\(^{2+}\)-injected side of immature or maturing oocytes. Vacquier (26) reports that 100-200-nm "struts of unknown nature" extend from sea urchin cortical granules to the plasma membrane and that isolated cortical granules break down when treated with Ca\(^{2+}\). When the breakdown of cortical granules was blocked with procaine (an agent that blocks Ca\(^{2+}\) binding in membranes [26]), they remained intact through cleavage and were found near the furrow. Thus, contraction and cortical granule breakdown were mechanistically separated, although both seem to depend on Ca\(^{2+}\) availability. It seems apparent from these and other experiments that calcium ions are important for changes such as contraction, cleavage, and cortical granule breakdown.

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