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Membrane Junctions in *Xenopus* Eggs: Their Distribution Suggests a Role in Calcium Regulation

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ABSTRACT We have observed the presence of membrane junctions formed between the plasma membrane and cortical endoplasmic reticulum of mature, unactivated eggs of *Xenopus laevis*. The parallel, paired membranes of the junction are separated by a 10-nm gap within which electron-dense material is present. This material occurs in patches with an average center-to-center distance of ~30 nm. These junctions are rare in immature (but fully grown) oocytes (~2% of the plasma membrane is associated with junctions) and increase dramatically during progesterone-induced maturation. Junctions in the mature, unactivated egg are two to three times more abundant in the animal hemisphere (25–30% of the plasma membrane associated with junctions) as compared with the vegetal hemisphere (10–15%). Junction density decreases rapidly to values characteristic of immature oocytes in response to egg activation.

The plasma membrane-ER junctions of *Xenopus* eggs are strikingly similar in structure to membrane junctions in muscle cells thought to be essential in the triggering of intracellular calcium release from the sarcoplasmic reticulum. In addition, the junctions' distinctive, animal-vegetal polarity of distribution, their dramatic appearance during maturation, and their disappearance during activation are correlated with previously documented patterns of calcium-mediated events in anuran eggs. We discuss several lines of evidence supporting the hypothesis that these junctions in *Xenopus* eggs are sites that transduce extracellular events into intracellular calcium release during fertilization and activation of development.

Alterations of intracellular calcium activity have been demonstrated to be important regulators of early developmental processes in the eggs of a large number of organisms (26, 45, 47). Although the source of intracellular calcium is unknown, it has been suggested that the endoplasmic reticulum (ER) might be involved in the regulation of calcium activity in a manner analogous to that of the sarcoplasmic reticulum (SR) of muscle cells (5, 15). In muscle cells calcium is sequestered in and released from regions of the SR which form specialized junctions with regions of the plasma membrane (12, 44). Although the mechanism by which these junctions act is still unknown, there is evidence that they somehow respond to a depolarization of the plasma membrane by triggering release of Ca^{2+} from the SR (1, 14, 16, 32, 36, 43). We have been investigating the role of the cortical ER of *Xenopus* eggs in the regulation of calcium activity and report the first observation in an egg of membrane junctions that are morphologically similar to the plasma membrane-SR junctions of muscle cells. We also report that these junctions in *Xenopus* eggs exhibit temporal and spatial patterns of distribution that are coincident

with previously documented patterns of calcium-mediated events.

MATERIALS AND METHODS

Gravid, female *Xenopus laevis* were purchased from Nasco Science (Fort Atkinson, WI). Full-grown (Stage VI) oocytes (7) along with their encapsulating follicle cells were removed with watchmaker forceps from the ovaries of decapitated females and were incubated in O-R2 saline (48), pH 7.8. Oocytes were induced to undergo meiotic maturation (42) in vitro by incubating them continuously in 4×10^{-6} M progesterone in O-R2 saline (20–22°C). Maturing oocytes were removed for fixation at various times during the following 9-h period. Unfertilized, mature eggs were obtained by injecting gravid females with 100 IU human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO), followed 36–48 h later with an additional 800 IU. Eggs were stripped from the animals 6–8 h after the latter injection.

For transmission electron microscopy, immature and maturing oocytes were fixed in ice-cold 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 90 min. In the case of mature, ovulated eggs, some were fixed immediately in ice-cold 6% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 90 min, whereas with most, the jelly layers first were removed by incubating the eggs in 2% cysteine in 27% DeBoer's saline (53), pH 7.2, for 3–5 min. Dejellied eggs were rinsed three times and incubated 10–30 min in 50% O-R2 saline (pH 7.8) before fixation in ice-cold 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 90

min. All samples were postfixed in ice-cold 1% OsO₄ in 0.1 M phosphate buffer (pH 7.2) for 60 min, dehydrated in ethanol, transferred to propylene oxide and embedded in Epon 812. Silver-gold interference sections were stained with saturated, aqueous uranyl acetate and lead citrate.

We quantified the density of plasma membrane-ER junctions on randomly selected and photographed regions of egg surface 15–20 μm in length. Regions of Stage VI oocytes and in vitro maturing oocytes from four different females, and mature, ovulated eggs from eight different females were examined. Because it is difficult to observe consistently the periodic electron-densities (Figs. 1–5), the criterion for identification of junctions was the parallel arrangement of the paired membranes with an intervening gap <20 nm. Since we never observed junctions in association with microvilli, we restricted our measurements to the intermicrovillar plasma membrane. In order to analyze the distribution of junctions, the meridians that connect the animal and vegetal poles of the egg were arbitrarily divided into major sectors, as follows: polar regions are within 250 μm of the respective poles; the equatorial region is within 125 μm of either side of the pigment transition between the animal and vegetal hemispheres; mid-hemisphere (animal or vegetal) regions are between the equatorial and polar regions (see Fig. 6).

RESULTS

Morphology of Plasma Membrane-ER Junctions

In mature, unactivated *Xenopus* eggs, cisternae of the cortical endoplasmic reticulum (ER) form distinctive junctions with the plasma membrane (Figs. 1–5). The junctional complex is characterized as follows: (a) ER and plasma membranes run parallel to each other along the length of the cistern. The extent of apposition of the paired membranes ranges from 100 nm to 700 nm (\bar{X} = 250 nm, N = 30). (b) The gap between the apposed membranes is narrow, ranging from 8–13 nm (\bar{X} = 10 nm, SD = 1.3, N = 25). (3) Periodic, electron-dense material is present in the junctional gap (Figs. 1–5, brackets). The average width of these densities is 17 nm (SD = 5 nm, N = 17) and the mean center-to-center spacing of adjacent densities is 30 nm (SD = 11 nm, N = 14). (4) The junctional ER cisternae lack associated ribosomes and occasionally are observed to be continuous with a previously reported network of ER deeper in the cortex (4) (Figs. 4 and 5).

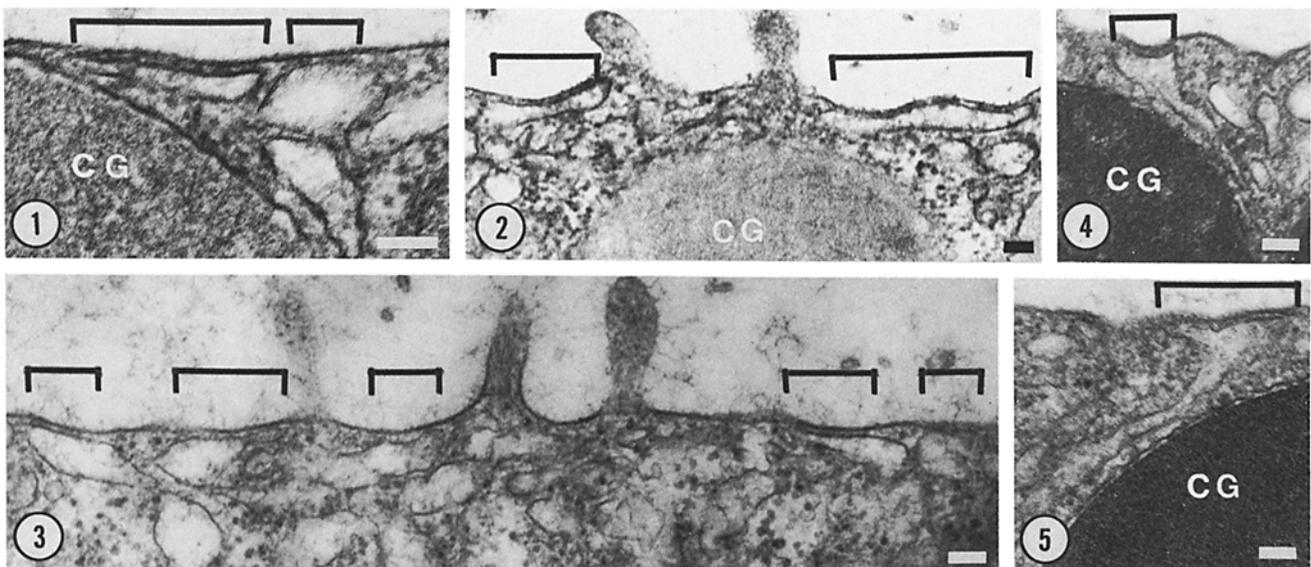
Spatial Distribution of Plasma Membrane-ER Junctions

The plasma membrane-ER junctions of *Xenopus* eggs exhibit two spatial patterns of distribution. (a) The junctions are

localized in the regions between microvillar projections on the egg surface. We presently do not have evidence indicating what, if any, the significance of this distributional pattern is; however, we have observed that the cores of some microvilli are occupied by microfilaments extending into the cortex (not shown in figures), presumably excluding ER cisternae from this region. (b) The junctions are two to three times more abundant in the animal hemisphere as compared to the vegetal hemisphere (Fig. 6). The values for the animal pole, middle animal hemisphere and equatorial regions are not significantly different from each other, but are significantly different from the values for the vegetal pole and middle vegetal regions, which in turn, are not significantly different from each other (P < 0.01; Newman-Keuls Least Significant Range Procedure [8]). The density of junctions within any particular region varies between oocytes, particularly in the equatorial region (standard deviation for equatorial values is 50–100% greater than for middle hemisphere and polar values). In order to eliminate “between-egg” variability, we quantified junction densities on large sections that extended from the middle animal region to the middle vegetal region. For the six eggs examined, there was no significant difference in junction density for one; in another, animal region junctions were eight times more abundant; in the other four eggs; animal hemisphere junctions were two to five times more abundant. On the average, junctions in these six eggs were 3.5 times more abundant in the animal hemisphere.

Increase in Junction Density During Maturation

The spatial pattern of plasma membrane-ER junctions develops during progesterone-induced maturation (Fig. 7). Junctions in fully grown, but immature oocytes (Stage VI) are rare as compared with mature, unactivated eggs. The increase in junction abundance during maturation is approximately five-fold in the vegetal hemisphere and 12-fold in the animal hemisphere. There is no increase in junction density within the initial 2 h of exposure to progesterone. Not all oocytes undergo in vitro maturation in response to progesterone treatment (34) (~30–50% underwent maturation in the present study). Since there are no visible changes in oocytes during the early phase



FIGURES 1–5 Electron micrographs of junctions between the plasma membrane and endoplasmic reticulum in unfertilized *Xenopus* eggs. Those regions of the junctions where periodic electron-densities are evident are indicated by brackets. In Figs. 4 and 5, the junctional ER cisternae are continuous with regions of the endoplasmic reticulum deeper in the cortex. CG, cortical granule. Bars, 0.1 μm. Fig. 1, × 80,000; Fig. 2, × 40,000; Fig. 3, × 47,000; Figs. 4 and 5, × 50,000.

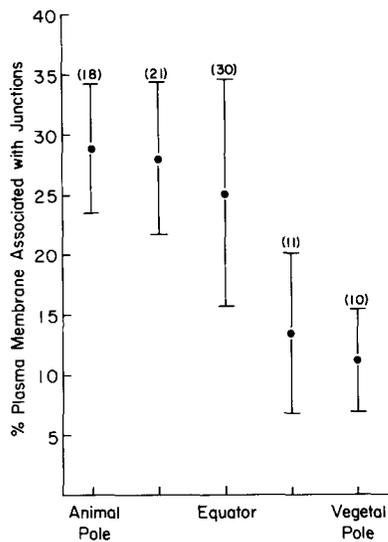


FIGURE 6 Percentage of the plasma membrane (between microvilli) forming junctions with endoplasmic reticulum in *Xenopus* eggs. Mean values are plotted, vertical lines represent one standard deviation, and number in parentheses is the number of regions examined.

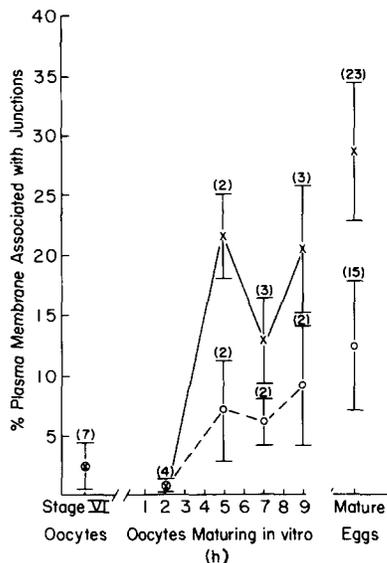


FIGURE 7 Percentage of the plasma membrane (between microvilli) forming junctions with endoplasmic reticulum in fully grown oocytes (Stage VI), in vitro maturing oocytes, and mature (oviposited) eggs of *Xenopus*. Mean values are plotted, vertical lines represent one standard deviation, and number in parentheses is the number of regions examined. Hours of in vitro maturation represent the time of continuous exposure to progesterone. (X), animal hemisphere regions; (O) vegetal hemisphere regions.

of maturation, we do not know whether the few oocytes we examined during the first 2-h period had, in fact, begun to respond to progesterone stimulation. At the time of the first observable indication of maturation (clearing of pigment at the animal pole indicating resumption of meiosis) at ~5 h of exposure to progesterone, junctions are present at densities comparable to those of mature, ovulated eggs.

Decrease in Junction Density During Egg Activation

Junction density decreases rapidly to values characteristic of immature oocytes in response to egg activation. When mature

eggs are activated by sperm, or by a variety of other stimuli, there is a progressive, wavelike exocytosis of cortical granules (cortical reaction) which takes 3–5 min to propagate around the egg (20, 21, 46). The decrease in junction density occurs at the time of cortical granule exocytosis. In TEM sections of the regions of the advancing wave of exocytosis in the animal hemisphere of two eggs, junction density in the regions that had not yet undergone the cortical reaction were 24% and 37%. In contrast, in those adjacent regions that had undergone the cortical reaction 30–60 s earlier (based on a propagation rate of 10 $\mu\text{m/s}$ [21]) junction densities were 8% and 2%. Values for the period from completion of the cortical reaction to first cleavage (8 min to 90 min post insemination) remained low ($\bar{X} = 2\%$, $SD = 2.4$, $N = 10$).

DISCUSSION

The plasma membrane-ER junctions of *Xenopus* eggs are strikingly similar in structure to membrane junctions thought to be the sites of excitation-contraction coupling in muscle cells. In muscle, the junctions are formed by an association of membranes of the sarcoplasmic reticulum either with invaginated regions of the plasma membrane or with localized, surface regions of the plasma membrane (13, 14, 22, 27), as in *Xenopus* eggs. Similar junctions also have been described for neurons and a variety of other cell types, in which they are hypothesized to function in the regulation of calcium activity (22, 23, 38, 39, 52). The junctions are characterized by the parallel arrangement of membranes with an intervening gap ranging from 5–20 nm. The 8–13 nm gap in *Xenopus* eggs is comparable to the 11–13 nm gap in skeletal muscle. The ER component of the junction lacks associated ribosomes in *Xenopus* eggs, muscle, and *Tetrahymena*, whereas ribosomes have been reported to occur occasionally in association with the junctional ER in the other cell types. Electron-dense material is present within the gap in *Xenopus* eggs, neurons, muscle cells, and *Tetrahymena*, but has not been reported for the other cell types. This material is either diffuse or somewhat periodic in neurons, but exhibits an interdensity spacing of ~20 nm in *Tetrahymena* and 30 nm in *Xenopus* eggs and muscle. In muscle, these electron-dense “feet” (13) or “pillars” (9) are thought to be essential structures in excitation-contraction coupling, and their fine structure and that of the associated junctional membranes have been extensively investigated. Results of different investigators presently are in conflict (compare references 13, 14, 16 with 29, 31, 32, 43), and thus there is no consensus as to structural details and functional mechanisms of the membranes and intermembranous material. To date we have not been able to resolve fine structural details of these junctions in *Xenopus* eggs; however, we presently are working to characterize more precisely the membranous and intermembranous components associated with the junctions.

In light of our present results, we hypothesize that plasma membrane-ER junctions in *Xenopus* oocytes are sites of calcium influx or release, with this calcium in turn stimulating calcium release from the associated ER. This hypothesis is based on proposed models of muscle plasma membrane-SR junction structure and function (1, 31, 32, 43) and is supported by four major lines of evidence pertaining specifically to eggs.

[1] During maturation there is an alteration of the calcium permeability of the plasma membrane and an increase in intracellular free calcium (33–35, 37, 51). Evidence supports the hypothesis that maturation involves changes in the cellular mechanism that controls free calcium concentration (24) and that this mechanism is compartmentalized and localized at or

near the plasma membrane (33, 50). These changes are reported to occur coincident with (34) or before the earliest time we could detect the formation of junctions. Caution is required in making temporal correlations of these phenomena, however, not only because of variability in response times exhibited by batches of eggs from different animals, but also because most ion transport studies have involved removal of immature oocytes from their follicles with the consequent disruption of follicle cell-oocyte gap junctions (3). Such treatment may result in ionic alterations not experienced in situ (49) and in possible damage to membrane ion transport mechanisms (34, 35). Even with these possible limitations, we feel it is significant to note that the plasma membrane in the animal hemisphere of defolliculated *Xenopus* oocytes apparently exhibits a higher calcium permeability as compared with the vegetal hemisphere (37). This polarity could be related to a higher density of plasma membrane microdomains that establish junctions with the cortical endoplasmic reticulum during maturation.

[2] The anuran egg cortex first acquires the ability to respond to activating stimuli during the process of oocyte maturation (2). The two major activation responses that have been well characterized—cortical granule exocytosis and a sustained membrane depolarization—are both calcium-dependent phenomena (6, 18, 24, 28, 41, 54). Preliminary experiments indicate that responsiveness to ionophore and pricking develop in the maturing *Xenopus* oocyte ~5 h after administration of progesterone, i.e., coincident with the appearance of the plasma membrane-ER junctions (Charbonneau, M., and R. D. Grey, unpublished observations). We have not yet determined the time of junction formation in other anuran oocytes, but note that onset of calcium-dependent responsiveness occurs late in the maturation process, as in *Xenopus* (2, 18, 25, 28, 40).

[3] The distribution pattern of plasma membrane-ER junctions correlates with the animal-vegetal polarity of calcium-dependent phenomena. (a) At fertilization, an increase in the chloride permeability of the plasma membrane results in a transient membrane depolarization (fertilization potential) (6, 19, 30). The fertilization potential can also be triggered by iontophoresis of Ca^{2+} , with the animal hemisphere responding 25–50 times more rapidly than the vegetal hemisphere (6). (b) A rise in intracellular, free Ca^{2+} at fertilization triggers the exocytosis of cortical granules beginning at the point of sperm entry into the egg and propagating around the circumference of the egg (11). Based on studies in a fish egg (medaka), it is inferred that the propagated wave of cortical granule exocytosis is triggered by a propagated wave of calcium-stimulated calcium release (15). The hypothesis of animal-vegetal polarized, calcium regulatory sites is supported by the observation that in anuran eggs, the wave of cortical granule exocytosis is propagated faster in the animal hemisphere as compared with the vegetal hemisphere (18). (c) The cortex of the newly activated egg undergoes an extensive contraction, presumably as a result of the rise in intracellular calcium (10, 17, 41, 46). This cortical contraction is greater in the animal hemisphere, as evidenced by a displacement of pigment toward the animal pole (46). A more rapid release and/or higher concentration of free calcium in the animal hemisphere could account for this phenomenon.

[4] The plasma membrane-ER junctions disappear in the wake of the cortical reaction. After the cortical reaction has occurred, eggs no longer exhibit a rapid membrane depolarization in response to electrical or mechanical stimulation (30), suggesting that intracellular calcium stores are no longer released.

To our knowledge, the results reported here are the strongest evidence to date for the presence in an egg of structures which, in other cell types, have been implicated repeatedly in the triggering of intracellular calcium release. These structures are present at a time and in a site appropriate to transduce extracellular events into intracellular calcium release, and they exhibit a polarized distribution coincident with known patterns of calcium activity regulation. We predict that similar sites are present and exhibit patterned distributions in other eggs that exhibit localized changes in free calcium concentration.

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