

# Cortical Localization of a Calcium Release Channel in Sea Urchin Eggs

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**Abstract.** We have used an antibody against the ryanodine receptor/calcium release channel of skeletal muscle sarcoplasmic reticulum to localize a calcium release channel in sea urchin eggs. The calcium release channel is present in <20% of immature oocytes, where it does not demonstrate a specific cytoplasmic localization, while it is confined to the cortex of all mature eggs examined. This is in contrast to the cortical and subcortical localization of calsequestrin in mature and immature eggs. Immunolocalization of the calcium release channel reveals a cortical reticulum or honeycomb staining network that surrounds cortical granules and is associated with the plasma membrane. The network consists of some immunoreactive electron-dense material coating small vesicles and elongate cis-

ternae of the endoplasmic reticulum. The fluorescent reticular staining pattern is lost when egg cortices are treated with agents known to affect sarcoplasmic reticulum calcium release and induce cortical granule exocytosis (ryanodine, calcium, A-23187, and caffeine). An ~380-kD protein of sea urchin egg cortices is identified by immunoblot analysis with the ryanodine receptor antibody. These results demonstrate: (a) the presence of a ryanodine-sensitive calcium release channel that is located within the sea urchin egg cortex; (b) an altered calcium release channel staining pattern as a result of treatments that initiate the cortical granule reaction; and (c) a spatial and functional dichotomy of the ER which may be important in serving different roles in the mobilization of calcium at fertilization.

**I**N sea urchin eggs increases in cytosolic calcium resulting from sperm or A-23187 activation (Steinhardt and Epel, 1974; Steinhardt et al., 1977) control processes such as cortical granule exocytosis, pronuclear migration, chromatin condensation, and nuclear envelope breakdown (Poenie et al., 1985; Steinhardt and Alderton, 1988; Twigg et al., 1988). Although egg activation is normally accompanied by a calcium influx, increases in intracellular free calcium can occur in the absence of external calcium (Nakazawa et al., 1970; Paul and Johnston, 1978; Fujino et al., 1985). Hence, mobilization of calcium from intracellular stores is sufficient to increase cytosolic calcium to a level capable of initiating egg activation and the cortical granule reaction.

The endoplasmic reticulum (ER) has been implicated as a storage site and regulator of calcium levels within eggs (Eisen and Reynolds, 1984; Turner et al., 1986; Oberdorf et al., 1988; Hensen et al., 1989). In addition to cisternae that are distributed throughout the cell's interior, a cortical network of ER, surrounding cortical granules and closely associated with the plasma membrane, has been observed in eggs from a variety of species (Gardiner and Gray, 1983; Sardet, 1984; Luttmer and Longo, 1985; Terasaki and Jaffe, 1991; Terasaki et al., 1991). Based primarily on structural similarities, it has been postulated that the cortical ER of

eggs functions in a manner similar to the sarcoplasmic reticulum of muscle cells (Gardiner and Gray, 1983; Sardet, 1984; Luttmer and Longo, 1985; Oberdorf et al., 1988; Henson et al., 1989).

Inositol trisphosphate (IP<sub>3</sub>)<sup>1</sup> induces calcium release from egg-derived ER, egg cortices, and whole eggs and may be the primary regulatory of intracellular calcium at fertilization (Whitaker and Irvine, 1984; Clapper and Lee, 1985; Oberdorf et al., 1986; Payan et al., 1986; Turner et al., 1986). However, a variety of other cells contain both IP<sub>3</sub>-sensitive and -insensitive calcium stores (Thayer et al., 1988a; Malgaroli et al., 1990; Wakui et al., 1990), the latter being sensitive to ryanodine and caffeine (Thayer et al., 1988a,b; Malgaroli et al., 1990) and stimulated by calcium-induced calcium release (CICR) (Nabauer et al., 1989; Wakui et al., 1990). In striated muscle, the ryanodine receptor is a high molecular weight tetramer (Lai et al., 1989), forming electron dense "feet" on the sarcoplasmic reticulum (Franzini-Armstrong, 1980). In both striated muscle and

1. *Abbreviations used in this paper:* ASW, artificial sea water; CaFASW, calcium-free artificial sea water; CIB, cortical isolation buffer; CIB-EGTA, cortical isolation buffer lacking EGTA; CICR, calcium-induced, calcium release; GTA, glutaraldehyde; IP<sub>3</sub>, inositol trisphosphate; PAF, paraformaldehyde; SWC, sea water C.

brain, the receptor functions as a calcium-, caffeine-, and ryanodine-sensitive calcium channel, suggesting that it is the gating mechanism for IP<sub>3</sub>-insensitive stores (Smith et al., 1988; Anderson et al., 1989; McPherson et al., 1991).

It has been proposed that eggs contain both IP<sub>3</sub>-sensitive and -insensitive calcium stores (Busa et al., 1985; Busa, 1990). Mouse and *Xenopus* oocytes demonstrate CICR (Berridge and Potter, 1990; Peres, 1990). Cyclic ADP ribose stimulates intracellular calcium release in sea urchin eggs from an IP<sub>3</sub>-independent calcium pool (Lee, 1991). Similarly, fertilization of sea urchin eggs induces intracellular calcium release despite the inhibition of IP<sub>3</sub>-mediated calcium stores (Rakow and Shen, 1990). Many known activators of the sarcoplasmic reticulum calcium release channel, including caffeine and ryanodine, induce calcium release in sea urchin eggs (Fujiwara et al., 1990; Galione et al., 1991; Buck, W. R., T. L. Rakow, and S. S. Shen. 1991. *J. Cell Biol.* 115:321a).

To identify and localize proteins potentially related to the calcium release channel of muscle cells, we have carried out studies demonstrating that a protein derived from isolated cortical preparations of sea urchin eggs immunoreacts with an antibody specific for the skeletal muscle ryanodine receptor. The protein is confined to the cortex of sea urchin eggs and forms a reticulum that is associated with the cortical granules and the plasma membrane. The reticulum consists of some immunoreactive electron-dense material that is associated with small vesicles and cisternae of ER. The reticulum's localization within the egg cortex and the ability of ryanodine to alter its staining pattern in isolated cortices demonstrate not only the presence of a calcium release channel but also a dichotomy in the ER of the sea urchin egg that may be important in mediating internal calcium levels at fertilization.

## Materials and Methods

### Animals

*Strongylocentrotus purpuratus*, *Lytechinus variegatus*, and *L. pictus* were obtained from Pacific Bio-marine Co. (Venice, CA) and Gulf Specimens (Panacea, FL) and maintained in a recirculating sea water aquarium.

### Cortex Preparation

Spawning was induced by intracoelomic injection of ~1 ml 0.5 M KCl. Pooled eggs were washed twice in artificial sea water (ASW; Ocean Aquariums, Mentor, OH), followed by homogenization at 4°C in sea water C (SWC; 0.5 M NaCl, 0.01 M KCl, 0.003 M NaHCO<sub>3</sub>, 0.03 M EGTA, and 0.06 M NaOH containing 100 µg/ml PMSF, 0.83 mM benzamide, and 1.1 µM leupeptin, pH 8.0; Kinsey, 1986) at a ratio of one volume of gravity-packed eggs to 10 volumes of SWC. The homogenate was diluted in 5 vol of SWC and spun at 2,000 rpm for 60 s. The pellet was washed gently in decreasing volumes (0.5×) of SWC and centrifuged. This washing procedure was continued until the cortex suspension was ~2 ml. The cortices were then pelleted and used for further analysis.

### SDS-PAGE and Immunoblot Analysis

Samples of eggs and pelleted cortices were analyzed by SDS-PAGE according to Laemmli (1970) on 3–12% gradient gels and stained with Coomassie blue or transferred to nitrocellulose sheets according to Towbin et al. (1979). Nitrocellulose transfers were stained according to McPherson and Campbell (1990) with polyclonal antibodies against the skeletal muscle ryanodine receptor.

## Antibody Production

Three antibodies to the skeletal muscle ryanodine receptor were prepared using the ascites method (Tung, 1983) as described by Sharp and Campbell (1989). Guinea pigs were injected intraperitoneally with skeletal muscle ryanodine receptor (10 µg; Imagawa et al., 1987) in Freund's complete adjuvant to induce the production of ascites fluid. Ascites fluid was tapped whenever significant amounts accumulated and was tested for antibody production by immunoblot analysis against purified skeletal muscle ryanodine receptor. Other antibodies that were tested included a rabbit antibody against a predicted A-kinase phosphorylation site of the cardiac ryanodine receptor (McPherson et al., 1991) and a sheep polyclonal against rabbit skeletal muscle ryanodine receptor (Knudson et al., 1990).

## Cortical Lawn Preparations

Eggs were washed in ASW, pH 5.0, to remove jelly coats, and allowed to settle on poly-L-lysine coated coverslips. Adherent eggs were sheared by a gentle stream of cortical isolation buffer (CIB; 0.6 M mannitol, 50 mM Hepes, 50 mM Pipes, 2.5 mM MgCl<sub>2</sub>, and 20 mM EGTA, pH 6.8; Henson and Begg, 1988) containing 100 µg/ml PMSF and monitored for the presence of cortical lawns. Lawns were prepared in a variety of fixatives: 3% paraformaldehyde/0.1% glutaraldehyde (3% PAF/0.1% GTA) in ASW (4°C) or calcium-free ASW (CaFASW) (4°C), 3% PAF in AC<sub>320</sub> (75 mM KCl, 2 mM MgCl<sub>2</sub>, 10 mM EGTA, 150 mM glycine, 320 mM sucrose, and 25 mM PIPES, pH 6.8, 4°C; Henson et al., 1989), 100% acetone (–20°C), or 3% PAF/0.1% GTA in CIB (20°C). Lawns, fixed for 20 min, were washed in the same buffer used for fixation, and then stored at –75°C or processed immediately for immunofluorescence microscopy. Some cortical lawns were left unfixed and prepared for dicarbocyanine- or immuno-staining.

To examine the possible involvement of the putative calcium release channel in the cortical granule reaction, cortical lawns of *L. variegatus* were incubated for 7–16 min with varying concentrations of ryanodine (100–2,000 µM; Penick Corp., Lindhurst, NJ), as well as other agents (100 µM calcium, 10 mM caffeine, and 10 µM A-23187) (Sigma Chemical Co., St. Louis, MO) known to stimulate cortical granule release. All agents were prepared in CaFASW, a calcium release buffer (0.3 M sucrose, 0.3 M KCl, 10 mM MgCl<sub>2</sub>, 30 mM Hepes, pH 7.2; Fujiwara et al., 1990) or CIB lacking EGTA (CIB-EGTA). Some cortical lawns were incubated simultaneously with 1 mM ruthenium red (Sigma Chemical Co.) and ryanodine. After treatment cortical lawns were left unfixed or fixed in 3% PAF/0.1% GTA in CaFASW or CIB and processed as described below for immunofluorescence microscopy. Cortical granule exocytosis was verified by phase microscopy.

## Immunofluorescence

Eggs, stratified eggs (Henson et al., 1989), and ovaries were fixed in 3% PAF in AC<sub>320</sub> or 3% PAF/0.1% GTA in ASW or CaFASW at 4°C for 2 h and washed in ASW or AC<sub>320</sub> overnight. AC<sub>320</sub> fixed specimens were processed as described by Henson et al., 1989). Fixed specimens were sectioned or maintained as whole mounts and permeabilized in 0.1% Triton X-100 in PBS (137 mM NaCl, 3 mM KCl, 6 mM NaHPO<sub>4</sub>, and 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 2 min) followed by a 5 min wash in PBS. Cryosections (5 µm) and whole mounts were blocked with 0.2% BSA in PBS or CIB (15 min) and incubated in ryanodine receptor antibody (1:50 to 1:150) for 2 h at 37°C. Specimens were washed twice in PBS or CIB (5 min each) followed by a 2-h incubation in the appropriate FITC-conjugated secondary antibody at 37°C. Cortical lawns were incubated in primary and secondary antibody for 15 min each at room temperature. After two 5 min washes in PBS or CIB, specimens were mounted in 0.1% *p*-phenylene diamine in glycerol, examined with an inverted microscope (Nikon Inc., Garden City, NY) equipped with fluorescence optics and photographed with TMAX 400 film (Eastman Kodak Co; Rochester, NY).

To demonstrate antibody specificity, specimens were incubated in: (a) the secondary antibody without primary antibody or (b) immuno-depleted ascites fluid. Ascites fluid was diluted 1:125 in 0.2% BSA in PBS and depleted of ryanodine receptor antibodies by incubation with nitrocellulose paper coated with purified skeletal muscle ryanodine receptor (Imagawa et al., 1987). Control nitrocellulose paper was coated with BSA. After a 2-h incubation, cryosections were reacted with processed ascites fluid and stained as described above.

## Dicarbocyanine Staining

Cortical lawns were prepared for dicarbocyanine (DiOC<sub>6</sub>[3]), Molecular

Probes, Eugene, OR) staining as described by Henson et al. (1989). Unfixed cortical lawns were stained for 1 min at room temperature in a 1:300 dilution in CIB of DiOC<sub>6</sub>(3) (2.5 mg/ml in 100% ethanol), washed in CIB, and examined immediately.

### **Transmission Electron Microscopy and Immunogold Labeling**

Eggs and stratified eggs fixed and washed as described above were permeabilized in 0.1% Triton X-100 in PBS followed by a 5 min wash in PBS and blocked for 15 min in 1% BSA in PBS. Specimens were incubated overnight at room temperature in ryanodine receptor antibody at 1:100, washed twice in PBS, and incubated in either protein A (Janssen Life Sciences Products; Piscataway, NJ) or anti-guinea pig antibody conjugated to 5-nm gold particles (Janssen Life Sciences Products) overnight at room temperature. Samples were washed twice in PBS followed by fixation in 3% GTA in PBS for 15 min, osmicated (0.5% OsO<sub>4</sub> in PBS) for 30 min at 4°C, and then dehydrated and embedded in Spurr's medium. Samples prepared solely for transmission microscopy were fixed in 3% GTA in 80% sea water, washed overnight in sea water, and dehydrated and embedded as described for immunopreparations. Thin sections were stained in uranyl acetate and lead citrate and examined in a Philips 300 (Philips Electronic Instruments, Mahwah, NJ) or Hitachi 7,000 (Hitachi Ltd., Tokyo) electron microscopes.

## **Results**

### **Immunofluorescence Localization of a Putative Calcium Release Channel**

Antibodies against striated muscle ryanodine receptor were initially screened for cross-reactivity to sea urchin egg proteins by immunoblot analysis. Of the three guinea pig antibodies tested, only one reacted positively and was subsequently used for all further studies. Antibodies made in rabbit were negative on immunoblots and were not tested on immunofluorescent preparations of sea urchin eggs. The sheep polyclonal tested positive with immunoblots of isolated sea urchin egg cortices, but was not as strongly reactive as the guinea pig polyclonal (data not shown). Immunostaining of permeabilized eggs, sections, and cortical lawns from the three species of sea urchins used in this study were virtually identical to one another, consequently only material from investigations with *L. variegatus* is used to illustrate the fluorescence localization of the calcium release channel/ryanodine receptor. Similar localization patterns were obtained from specimens prepared in the various fixatives outlined in Materials and Methods.

The specificity of immunostaining was determined using sectioned eggs incubated with immunodepleted ascites fluid (Fig. 1, *a* and *b*). Sections reacted with ascites fluid incubated with BSA-coated nitrocellulose were positive (Fig. 1 *a*), whereas those reacted with ascites fluid formerly incubated with nitrocellulose containing highly purified skeletal muscle ryanodine receptor were negative (Fig. 1 *b*). That is, the latter lacked the bright, cortical staining seen with complete and BSA-treated ascites fluid. These sections directly demonstrate the specificity of the antibody-antigen reaction and suggest that the reactive product is a calcium release channel similar to that found in skeletal muscle (Smith et al., 1988).

Permeabilized eggs reacted with the ryanodine receptor antibody demonstrated bright staining deposits that were absent in preparations lacking the primary antibody (data not shown). Through-focus of permeabilized eggs revealed that the staining was confined primarily to the egg's cortex. Sectioned specimens reacted with the antibody demonstrated a

cortical staining along the entire periphery of the egg, consisting of small deposits of fluorescence (Fig. 1, *c* and *e*). In grazing sections of the cortex, the staining had a honeycomb appearance, i.e., it consisted of a reticulum of interconnected circular staining areas (Fig. 1 *e*). The width of the zone of cortical staining measured  $\sim 5 \mu\text{m}$ ; staining was not apparent deeper to this zone (Fig. 1, *c* and *e*), or in preparations incubated only in the secondary antibody (Fig. 1 *d*).

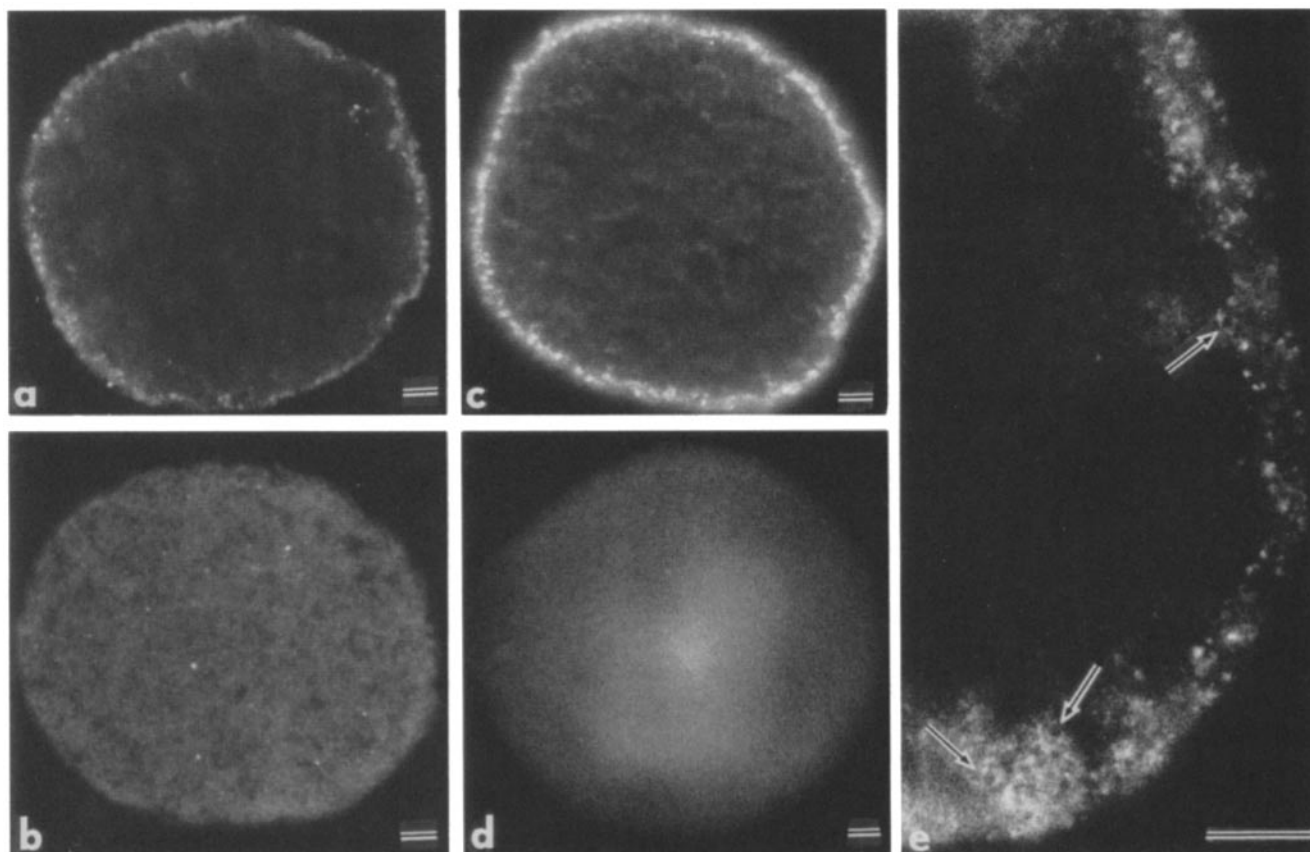
Unfixed or fixed cortical lawn preparations were essentially identical and demonstrated a reticular staining pattern with the ryanodine receptor antibody (Fig. 2 *a*). Occasionally, a pattern consisting of densely packed small circles was also observed, due possibly to variations in cortical lawn preparation. In both sections (Fig. 1 *e*) and cortical lawns (Fig. 2, *a* and *b*), the staining pattern appeared to circumscribe circular non-staining areas  $\sim 1 \mu\text{m}$  in diameter, suggesting that the calcium release channel was organized around individual cortical granules. Staining of unfixed cortical lawns with the membrane penetrating dye, DiOC<sub>6</sub>(3), also revealed a reticular network that surrounded cortical granules (Fig. 2, *d* and *e*) similar to patterns observed by Henson et al. (1989) and Terasaki et al. (1991). The organization of the calcium release channel/ryanodine receptor and cortical granules was verified by immunoelectron microscopy (see below).

In contrast to the distinct cortical staining with antibody to ryanodine receptor, antibodies to calsequestrin reacted with components throughout the egg's interior (data not shown; see Henson et al., 1989). Stratified eggs of *L. variegatus* stained with antibody to the ryanodine receptor showed a similar pattern to that described for specimens that were not centrifuged, i.e., reaction product was confined to the egg cortex (data not shown). In contrast, when stratified eggs were reacted with antibodies to calsequestrin, fluorescent reaction product was distributed within the region (clear area) surrounding the female pronucleus (data not shown; see Henson et al., 1989).

Sections of *L. variegatus* ovary were stained with the ryanodine receptor antibody to determine the localization of the calcium release channel/ryanodine receptor in immature oocytes. Most oocytes did not stain (Fig. 3 *a*). However, a few (<20%), displayed sparse fluorescence which was not necessarily confined to the oocyte cortex (data not shown). We suspect that differences in immunoreactivity of immature oocytes is reflective of different stages of oogenesis and further studies are underway to verify this suspicion. In contrast, all immature eggs demonstrated a positive staining reaction with calsequestrin antibodies throughout their cytoplasm (data not shown; see Henson et al., 1989).

### **Electron Microscopic Localization of the Calcium Release Channel**

Permeabilized eggs of *L. pictus*, *L. variegatus*, and *S. purpuratus*, prepared for colloidal gold localization, were labeled along their cortices in a manner similar to that which was seen in fluorescent preparations (Figs. 4 and 5). Colloidal gold particles were localized to small vesicles and elongate cisternae that surrounded the cortical granules and were positioned subjacent to the plasma membrane (Figs. 4 and 5). In appropriate sections of the egg cortex, in which the lumens of vesicles and elongate cisternae were clearly dis-



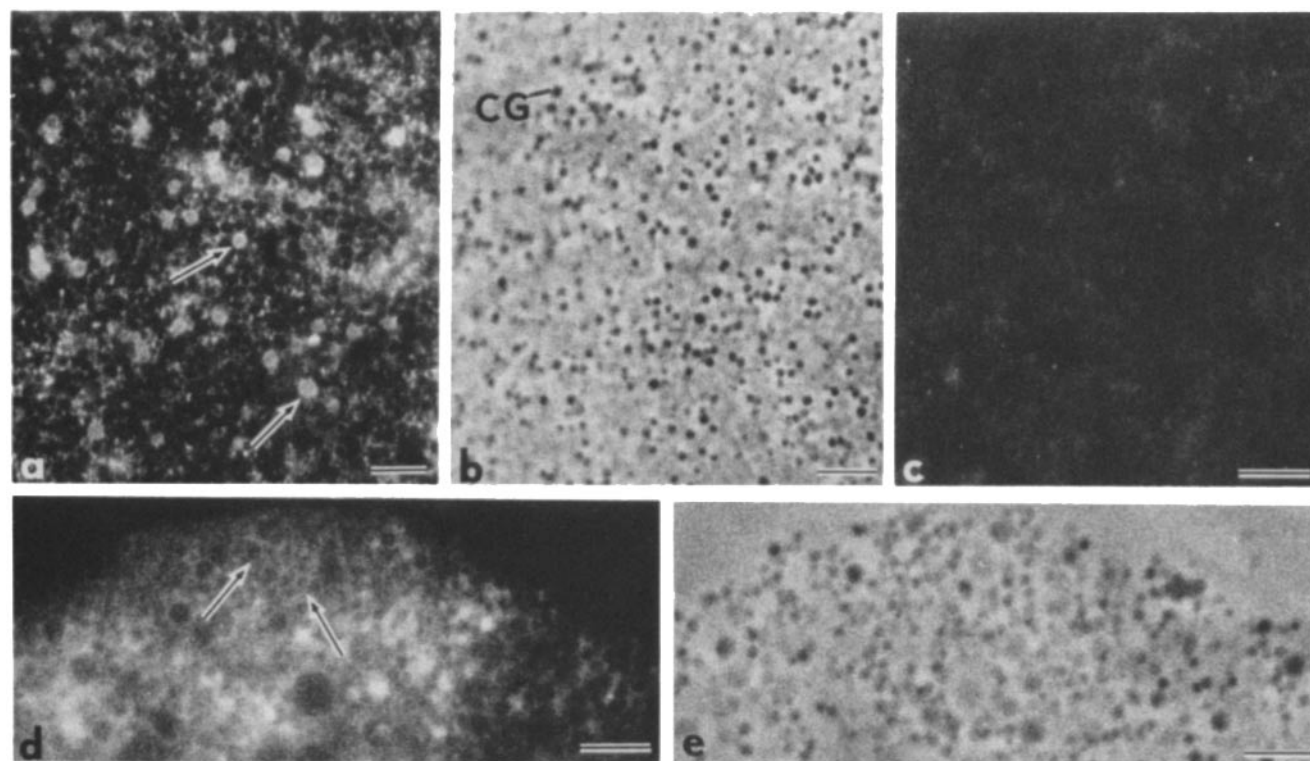
**Figure 1.** Immunofluorescent preparations of *L. variegatus* eggs (fixed in 3% PAF in AC<sub>320</sub>) demonstrate cortical localization of the calcium release channel. (a and b) Sections stained with ascites fluid that was first incubated with nitrocellulose strips coated with BSA (a) or highly purified skeletal muscle ryanodine receptor (b). Reaction product to the ryanodine receptor antibody is confined to the egg cortex (a). Immunoreactive material is not associated with the section stained with immunodepleted ascites fluid (b) demonstrating the specificity of the calcium release channel/ryanodine receptor antibody. (c–e) Sections stained with (c and e) or without (d) ryanodine receptor antibody demonstrating that the staining product is confined to the egg's cortex. The staining deposits in sectioned eggs (e) are occasionally seen to form a reticulum (arrows) that surrounds unstained circular areas that may correspond to cortical granules. Bars, 10  $\mu\text{m}$ .

cerned, a definite labeling pattern was apparent. That is, gold particles were associated with some electron-dense material along the outer aspect of membranes delimiting labeled vesicles and elongate cisternae (Fig. 5, a and b). Label was not found localized to other organelles nor was it associated with cisternae of ER in subcortical regions of the egg (Fig. 4 b). Colloidal gold label was absent entirely in preparations reacted only with secondary antibody (Fig. 5 c). In control specimens (Fig. 5 c), and in specimens not prepared for immunogold staining (Fig. 5 d), cortical granules were surrounded and the plasma membrane was subtended by a network of small vesicles and elongate cisternae which in turn were associated with some electron-dense material. Additionally, the elongate cisternae possessed ribosomes (Fig. 5 d).

#### **Agents That Induce Calcium Release Alter the Localization of the Calcium Release Channel in Cortical Lawns**

In an effort to examine the potential role of the calcium release channel at fertilization, and in the initiation of the cortical granule reaction, cortices were incubated in ryanodine and other agents (caffeine, calcium, A-23187, and ruthenium red) known to affect sarcoplasmic reticulum calcium release and cortical granule exocytosis (Steinhardt and Epel, 1974;

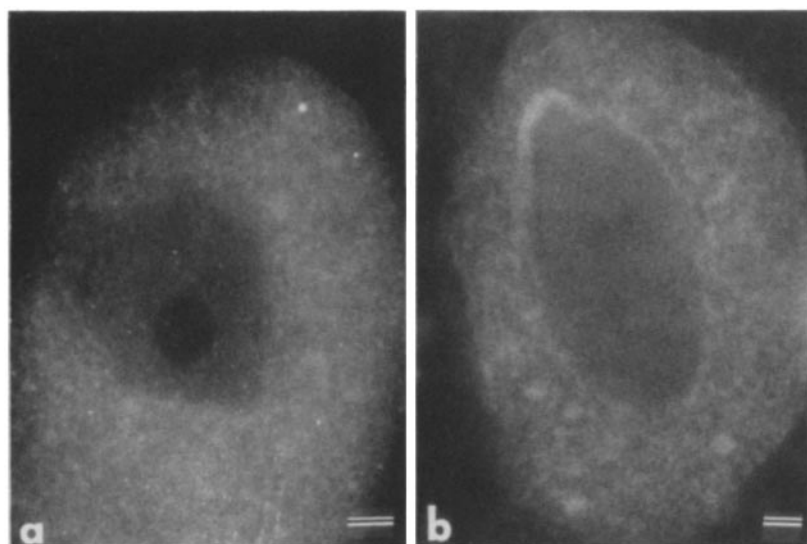
Steinhardt et al., 1977; Fujiwara et al., 1990). The cortices were either left unfixed or fixed and stained with the ryanodine receptor antibody to examine possible changes in the localization of the calcium release channel. The resulting staining patterns were essentially identical in unfixed and fixed preparations indicating that the methods used here did not affect calcium release channel/ryanodine receptor localization. Cortices incubated in CIB-EGTA (Fig. 2, a and b), CaFASW, AC<sub>320</sub>, or a calcium release buffer (Fujiwara et al., 1990) alone or in the vehicle to solubilize ryanodine (5% ethanol; data not shown) did not undergo cortical granule release and yielded a bright reticular fluorescent pattern when stained with antibody to the ryanodine receptor. By phase contrast microscopy, cortical granules were seen as a lawn of dense spheroids (Figs. 2 b and 6 d; Whitaker and Baker, 1983; Chandler, 1984). In contrast, large areas of cortices reacted with 1 mM ryanodine (Fig. 6, a and b), 10  $\mu\text{M}$  A-23187 (Fig. 6, e and f), 100  $\mu\text{M}$  calcium (Fig. 6, g and h), or 10 mM caffeine (data not shown) were essentially devoid of cortical granules and demonstrated a lack or decrease in reticular staining with the ryanodine receptor antibody. Cortices incubated simultaneously with ryanodine and 1 mM ruthenium red (Fig. 6, c and d), a specific blocker of the sarcoplasmic reticulum calcium release channel (Imagawa et



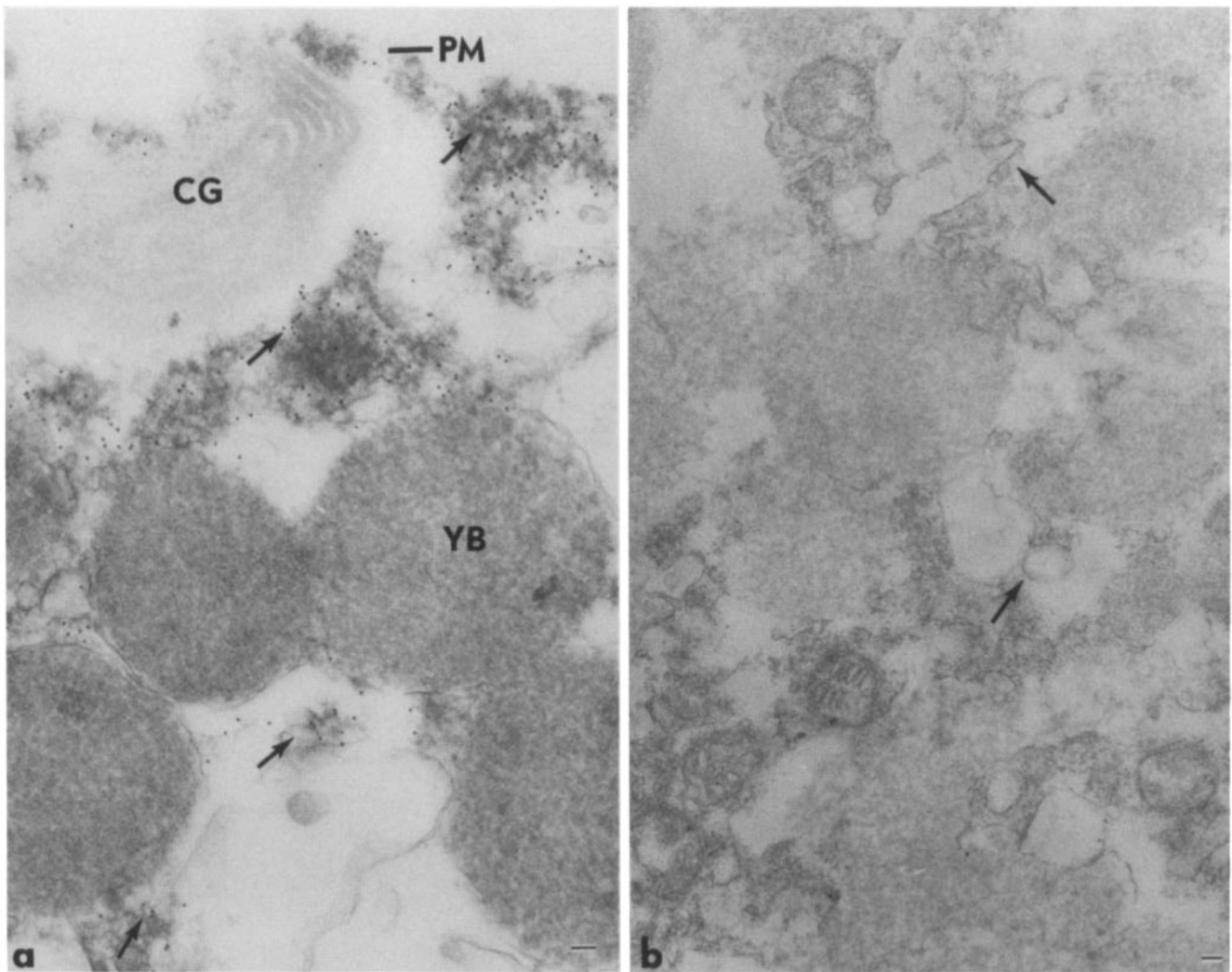
**Figure 2.** Cortical lawns demonstrate a reticular pattern when stained with the ryanodine receptor antibody or with the membrane penetrating dye, DiOC<sub>6</sub>(3). (*a* and *b*) Fluorescent and phase micrographs of the same cortical lawn preparation (fixed in 3% PAF/0.1% GTA in CIB) in which the ryanodine receptor localization reveals a honeycomb pattern that appears to circumscribe cortical granules (CG). It is noteworthy that diameters of the open or unstained areas of the honeycomb are about the same size as diameters of the cortical granules. The solid circular staining areas indicated by arrows (*a*) are believed to represent cortical granules that are surrounded by ryanodine receptor positive components (vesicles and elongate cisternae) of the egg cortex. (*c*) Staining is absent in control preparations lacking the primary antibody. (*d*) Unfixed cortical lawns stained with DiOC<sub>6</sub>(3) reveals a fluorescent reticulum similar to that obtained with ryanodine receptor antibody staining, i.e., a cortical honeycomb network (*arrows*). (*e*) Phase contrast image corresponding to *d* demonstrating phase-contrast cortical granules. Bar, 5 μm.

al., 1987), demonstrated a labeling pattern similar to that of untreated lawns (Fig. 2 *a*), and did not exhibit cortical granule exocytosis (Fig. 2 *b*). These data provide strong evidence for: (*a*) the release of calcium from intracellular stores of cortical lawn preparations resulting in cortical granule loss; and

(*b*) a ryanodine, caffeine, and ruthenium red-sensitive calcium release mechanism. This and our observations demonstrating an immunoreactive protein similar to the muscle ryanodine receptor support the hypothesis that a ryanodine-sensitive calcium release channel exists in sea urchin eggs.



**Figure 3.** Immature eggs, deficient in the calcium release channel, do not display cortical staining. Ovarian sections of immature oocytes (fixed in 3% PAF in AC<sub>320</sub>) of *L. variegatus* stained with (*a*) or without (*b*) antibody to ryanodine receptor. Germinal vesicle oocytes demonstrate very little or no staining when compared to mature eggs. Bars, 10 μm.



**Figure 4.** The calcium release channel is localized to small vesicles and cisternae associated with cortical granules and the plasma membrane. (a) Cortex of *L. variegatus* in which 5-nm colloidal gold particles are associated with some electron-dense material coating small vesicles and cisternae (arrows) that surround cortical granules (CG) and yolk bodies (YB), and subtend the plasma membrane (PM). (b) Very few gold particles are found associated with elements of the ER (arrows) in subcortical regions of the egg. Bars, 0.1  $\mu\text{m}$ .

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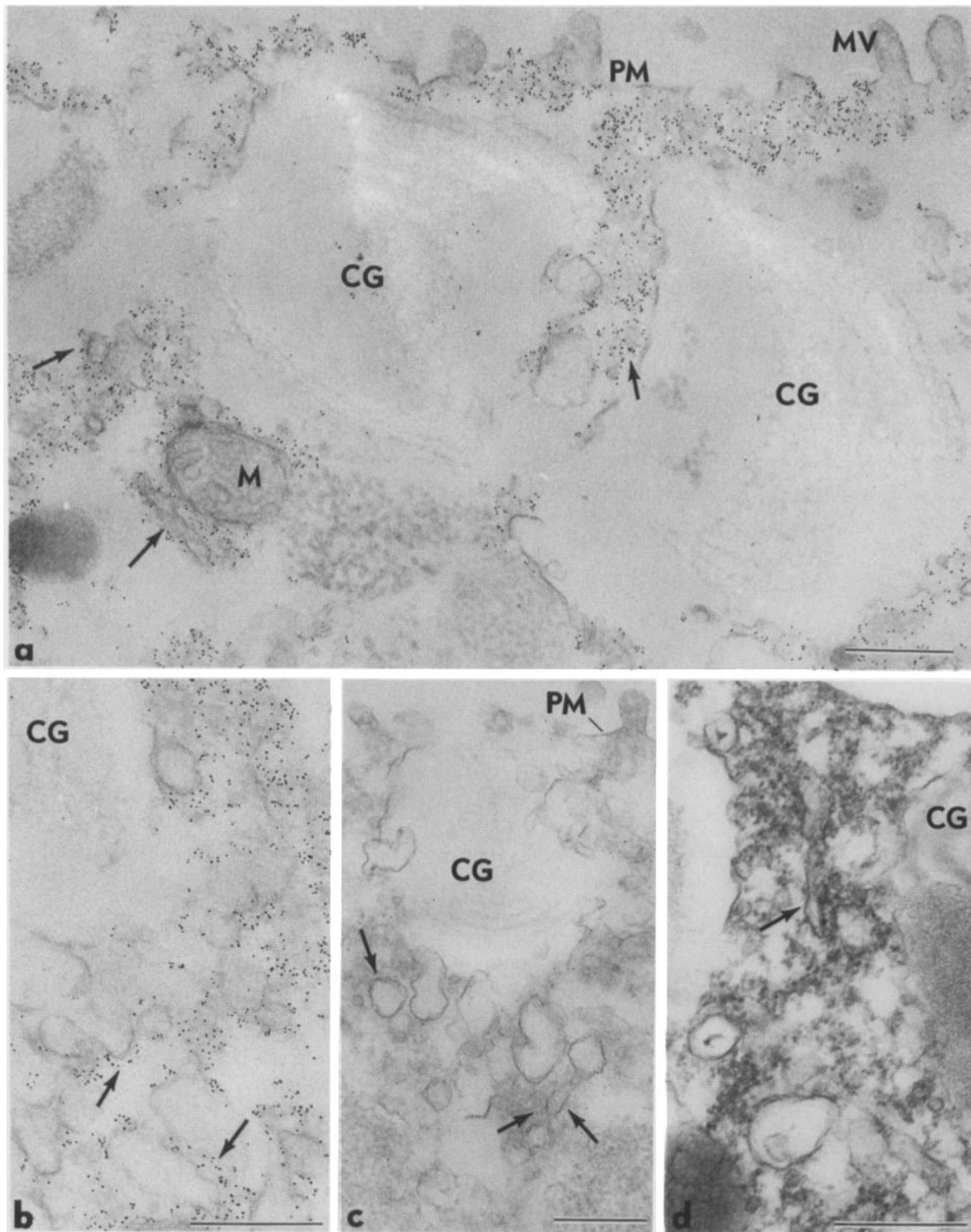
### Immunoblot Identification

Cortices prepared from *L. variegatus* and analyzed by SDS-PAGE were highly enriched in numerous protein bands, particularly at  $\sim 165$  kD and in excess of 200 kD when compared to whole egg homogenate (Fig. 7 a). One of the bands in the cortical preparation, at a molecular weight of  $\sim 380$  kD, was stained on immunoblots with the ryanodine receptor antibody (Fig. 7 b) generated in guinea pig. The band also reacted with a sheep polyclonal antibody against the mammalian skeletal muscle ryanodine receptor (data not shown). Because the immunoreactive protein is located within the egg cortex, considerable enrichment was necessary to detect it on immunoblots (Fig. 7 b).

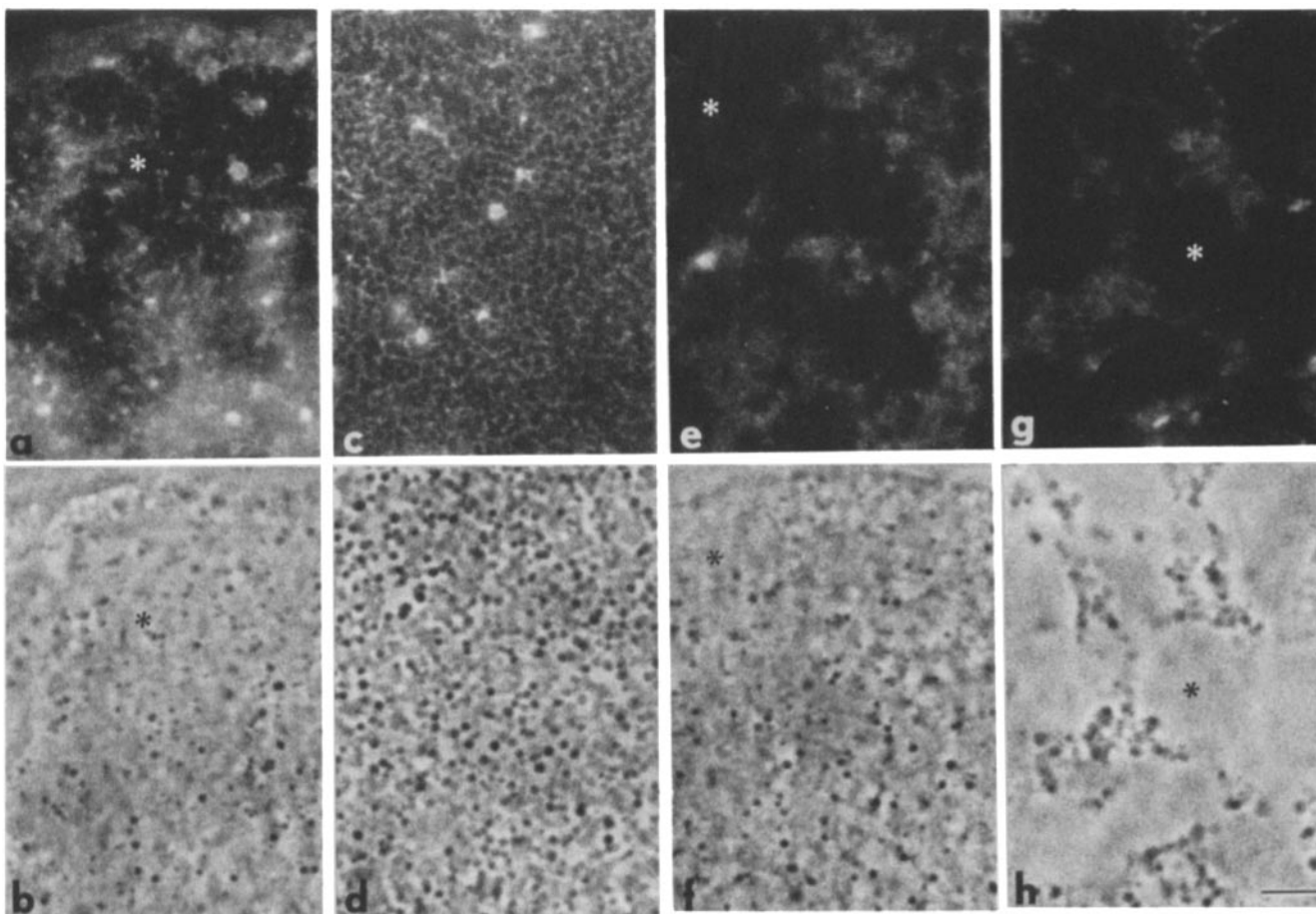
### Discussion

Calcium is one of the principal messengers in the regulation of fertilization events in sea urchin eggs (Whitaker and Patel, 1990).  $\text{IP}_3$ -induced calcium release from intracellular stores

has been implicated as a primary regulator of intracellular calcium levels (Turner et al., 1986). However, sea urchin eggs also contain  $\text{IP}_3$ -insensitive pools of calcium (Lee, 1991) which are sensitive of caffeine and ryanodine (Fujiwara et al., 1990; Galione et al., 1991) and may, therefore, be gated by a ryanodine receptor similar to skeletal muscle and brain (Smith et al., 1988; McPherson et al., 1991). While there is pharmacological evidence for  $\text{IP}_3$ -insensitive calcium pools in sea urchin eggs, their localization and the presence of a ryanodine receptor have not been demonstrated. Using antibodies prepared against the native skeletal muscle ryanodine receptor/calcium release channel, we demonstrate the presence and location of a high molecular weight calcium release channel in eggs of *Lytechinus pictus*, *L. variegatus*, and *Strongylocentrotus purpuratus*. That all of the antibodies to skeletal muscle ryanodine receptor tested did not cross-react with any sea urchin proteins strengthens the reliability and specificity of the guinea pig and sheep antibodies which demonstrate a positive reactivity to the ryanodine receptor/calcium release channel. The



**Figure 5.** Ryanodine receptor antibody staining is localized to a network of vesicles and elongate cisternae within the egg cortex. Permeabilized *S. purpuratus* eggs reacted with (a and b) or without (c) ryanodine receptor antibody, followed by secondary antibody conjugated to 5 nm colloidal gold. (a) Colloidal gold particles are associated with some electron-dense material that surrounds elongate cisternae and vesicles (arrows) that outline the egg's cortical granules (CG) and fill regions between cortical granules and the plasma membrane (PM). (b) Higher magnification of an egg cortex demonstrating the association of colloidal gold with the surface of small vesicles and cisternae (arrows) that surround the cortical granules (CG). (c) Section, in which the primary antibody was omitted, showing a lack of staining with the colloidal gold second antibody. Vesicles and elongate cisternae which are embedded in some electron-dense material (arrows) are associated with cortical granules (CG) and the plasma membrane (PM). (d) Ultrastructure of the egg cortex demonstrating elongate cisternae and vesicles of the ER (arrow). M, mitochondria; MV, microvilli. Bars, 0.5  $\mu$ m.



**Figure 6.** Agents (ryanodine, A-23187, and calcium) which affect sarcoplasmic reticulum calcium release and induce cortical granule exocytosis alter ryanodine receptor antibody staining of cortical lawns. (a, c, e, and g) *L. variegatus* cortical lawns stained with ryanodine receptor antibody after treatment with 1 mM ryanodine (a), 1 mM ryanodine plus 1 mM ruthenium red (c), 10  $\mu$ M A-23187 (e), or 100  $\mu$ M calcium (g) in CIB-EGTA. Immediately after treatment, cortical lawns were fixed in 3% PAF/0.1% GTA in CIB. (a, e, and g) In treatments which elicit cortical granule exocytosis, the labeling pattern is altered. That is, in contrast to the bright reticular staining pattern of untreated preparations (see Fig. 2 a) or specimens incubated with ryanodine plus ruthenium red (c), areas having undergone cortical granule exocytosis show a decrease or lack of staining with the ryanodine receptor antibody (asterisks). Areas lacking cortical granules are indicated by asterisks in the corresponding phase contrast micrographs (b, f, and h). (d) Phase contrast micrograph that corresponds to the fluorescent image shown in Fig. 6 c. Bars, 5  $\mu$ m.

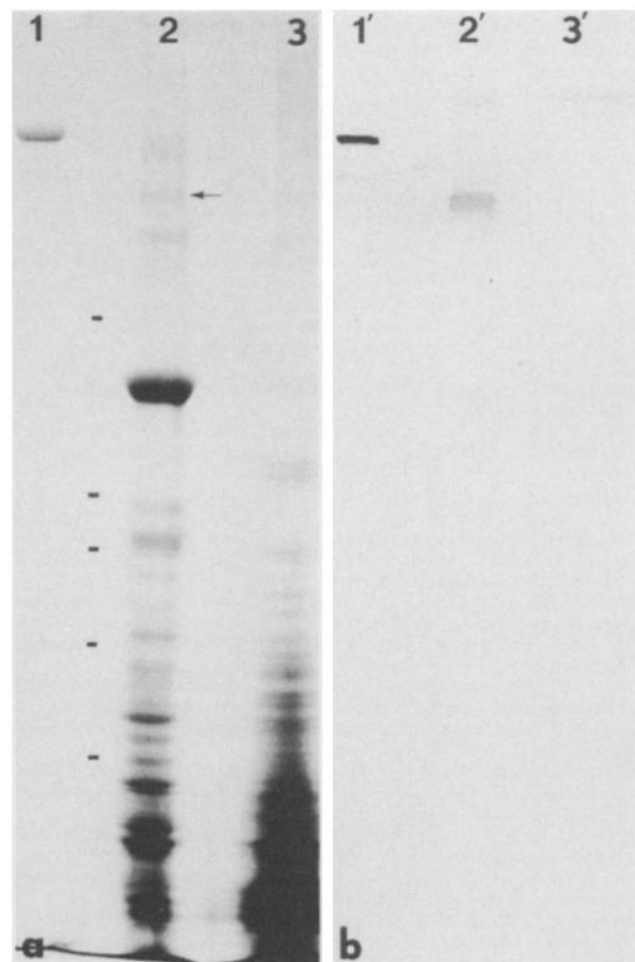
negative results of sections stained with ascites fluid immunodepleted using highly purified skeletal muscle ryanodine receptor demonstrate the specificity of the antibody preparation employed here. The calcium release channel of sea urchin eggs has a smaller apparent molecular weight ( $\sim$ 380 kD) than that of rabbit skeletal muscle or brain ryanodine receptors but is similarly enriched in membrane preparations. Additionally, micromolar amounts of ryanodine are required to stimulate calcium release from sea urchin egg microsomes (Fujiwara et al., 1990) indicating that the sea urchin egg ryanodine receptor has a lower affinity for ryanodine than that in mammals.

As shown here the ryanodine receptor is localized to elongate cisternae and vesicles which form a reticulum around the cortical granules and correspond to membrane networks discerned by other methods (Sardet, 1984; Terasaki and Jaffe, 1991; Terasaki et al., 1991). Based on previous studies in sea urchin eggs (Sardet, 1984; Chandler, 1984; Luttmner and Longo, 1985), we believe the labeled vesicles and elongate cisternae seen here are not independent structures but

actually represent tubular elements of the cortical ER possessing varicosities along their lengths. This network then, is viewed as a component of the egg's ER, rather than a separate, specific organelle, such as calciosomes (Rossier and Putney, 1991).

In stratified eggs, calsequestrin staining is predominantly confined to the clear layer (Henson et al., 1989), a region to which the bulk of ER becomes confined during isopycnic centrifugation (Harvey, 1956; Anderson, 1968), whereas the calcium release channel is present in elements of the ER that remain associated with the cortical granules and plasma membrane (Luttmner and Longo, 1985). These data, recent observations of Terasaki and Jaffe (1991) and Terasaki et al. (1991) and that reported here indicate that the ER of the sea urchin egg consists of two distinct structural domains and possibly, two functional domains, as well. One, the cortical ER, which is associated with the plasma membrane, surrounds cortical granules, and possesses the calcium release channel/ryanodine receptor. The other consists of a subcortical cisternal network of ER that courses throughout the cell interior and





**Figure 7.** Ryanodine receptor antibody recognizes a high molecular weight protein in *L. variegatus* cortical preparations. 3–12% SDS-polyacrylamide gel (a) and corresponding immunoblot (b) probed with antibody to the skeletal muscle ryanodine receptor protein. Lane 1, purified skeletal muscle ryanodine receptor protein; lane 2, *L. variegatus* cortices; and lane 3, *L. variegatus* eggs. The skeletal muscle ryanodine receptor shows an intense reaction with the antibody (lane 1'). A band that reacts with the antibody is also present in the sea urchin cortex preparation (lane 2') which correspond to a band(s) depicted by the arrow in the gel (a). The positions of molecular weight markers are indicated by dashes in between lanes 1 and 2, from top to bottom: 205, 115, 97, 67, and 45 kD.

is apparently deficient in the calcium release channel/ryanodine receptor.

The lack of the calcium release channel in sea urchin oocytes amplifies previous observations demonstrating that immature eggs do not undergo a cortical granule reaction (Harvey, 1956; Longo, 1978; Charbonneau and Grey, 1984). With egg maturation, changes in the ER occur (Henson et al., 1990) which may be necessary for the egg to undergo a cortical granule response. In addition to the localization of the cortical granules to the egg plasma membrane (Longo, 1978), the ER may acquire calcium release protein. Investigations by Chiba et al. (1990) have demonstrated that for a given stimulus more calcium is released from starfish eggs than oocytes. Such observations are consistent with the suggestion that the cortical ER contains a calcium release chan-

nel which is essential for egg activation and cortical granule release.

We find that agents known to affect sarcoplasmic reticulum calcium release and cortical granule exocytosis, i.e., calcium (Steinhardt et al., 1977), A-23187 (Steinhardt and Epel, 1974; Oberdorf et al., 1986; Terasaki and Sardet, 1991), ryanodine, and caffeine (Fujiwara et al., 1990; Galione et al., 1991), induce cortical granule loss and a change in the localization pattern of the calcium release channel in cortical lawns. These changes are consistent with observations in which: (a) the cortical ER of fertilized eggs demonstrates a more diffuse staining pattern than that of unfertilized eggs (Henson et al., 1989; Terasaki and Jaffe, 1991); (b) lawns treated with calcium undergo a loss of cortical granules (Vacquier, 1975; Whitaker and Baker, 1983; Chandler, 1984); and (c) lawns treated with A-23187 release calcium from intracellular stores (Oberdorf et al., 1986; Terasaki and Sardet, 1991). That the changes reported here are stimulated by ryanodine and inhibited by ruthenium red suggests a specific role for the calcium release channel/ryanodine receptor in cortical granule exocytosis.

It is noteworthy that at the electron microscopic level, colloidal gold localization within the egg cortex was distinguished by its association with some electron-dense material coating elements of the ER. This labeling pattern is similar to the localization of the ryanodine receptor in rough and smooth ER of brain and muscle (C. M. Knudson and K. Campbell, unpublished observations; Ellisman et al., 1990; Walton et al., 1991). It would be of considerable interest to determine whether or not the electron-dense material associated with the ER elements labeled here is analogous to the "feet" structures of the sarcoplasmic reticulum (Franzini-Armstrong, 1980) and brain (McPherson et al., 1991). Virtually identical immunofluorescent staining patterns in unfixed and fixed cortical lawns suggest that epitopes of the ryanodine receptor are present along the cytoplasmic surface of the sea urchin cortical ER and that the egg ryanodine receptor may be organized in a manner similar to that proposed for the skeletal muscle calcium release channel (Takeshima et al., 1989).

Based on its structural similarity to junctions formed by the sarcoplasmic reticulum and T-tubules in skeletal muscle (Franzini-Armstrong, 1980), it has been proposed that sites of association of the cortical ER with the egg plasma membrane may regulate intracellular-free calcium concentration at egg activation (Gardiner and Grey, 1983; Sardet, 1984; Charbonneau and Grey, 1984; Luttmner and Longo, 1985). At activation, a sodium-dependent depolarization of the egg plasma membrane could stimulate voltage-sensitive calcium channels on the egg surface (Hagiwara and Jaffe, 1979), which in turn may associate with ryanodine receptor-like protein at cortical ER-plasma membrane junctions (Jaffe, 1983). However, as shown here the system of ER that labels with the anti-ryanodine receptor antibody extends deeper into the egg cortex than would be expected for a junction postulated to be structurally similar to the triad.

It has also been proposed that sperm-egg interaction results in the release of IP<sub>3</sub> through G protein activation which induces a local increase in internal calcium (Busa et al., 1985; Turner et al., 1986). This in turn causes a CICR, which is a more extensive release of calcium and is propagated via further CICR (Galione et al., 1991). CICR is believed to be involved in cortical granule exocytosis (Busa et

al., 1985; Peres, 1990; Busa, 1990). The localization of the calcium release channel to sea urchin egg cortices adds to this model, giving it a spatial context in which its components may function. For example, it is possible that the CICR is derived from vesicles and cisternae located in association with the cortical granules much like that of the sarcoplasmic reticulum. Such a suggestion is consistent with results of experiments demonstrating that there are at least two pools of stored calcium in eggs, IP<sub>3</sub> sensitive and insensitive (Busa et al., 1985; Turner et al., 1986; Fujiwara et al., 1990; Peres, 1990; Lee, 1991; Galione et al., 1991). How the ER domains observed here (see also Terasaki and Jaffe, 1991) are related spatially and functionally and are involved in the propagation of calcium waves at fertilization requires further elucidation.

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