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Characterization of Sea Urchin Egg Endoplasmic Reticulum in Cortical Preparations

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The cortical endoplasmic reticulum (ER) of sea urchin eggs was localized on isolated egg cortices by staining with aqueous suspensions of the dicarbocyanine "DiI." Immunofluorescence localization of a calsequestrin-like protein was essentially identical; this is consistent with a role for the ER in calcium regulation. The ER often encircles cortical granules, making it well-suited for initiating fusion and propagating the calcium wave. Thiazole orange and Hoechst dye 33258 at pH 2 stain ribosomes bound to the ER, providing evidence that the cortical ER is rough ER. High chloride concentrations were found to disrupt ER continuity.

INTRODUCTION

The endoplasmic reticulum (ER) of the sea urchin egg consists primarily of a cortical network and interior cisternae (Terasaki and Jaffe, 1991). ER membranes are the site of protein and lipid synthesis and may be the principal source of calcium at fertilization (Eisen and Reynolds, 1985; Clapper and Lee, 1987; Han and Nuccitelli, 1990). At present, studies of ER function are hampered by difficulties in isolating ER. Homogenization/differential centrifugation is a lengthy, disruptive procedure that also does not produce pure ER membranes.

Intact elements of the cortical ER were observed on fixed sea urchin egg "cortices" by electron microscopy (Sardet, 1984; Chandler, 1984) and by immunofluorescence to a calsequestrin-like protein (Henson *et al.*, 1989). The dicarbocyanine "DiI" stains a similar network on unfixed cortices (Terasaki *et al.*, 1988; Henson *et al.*, 1989). Here, staining of the ER by DiI is described in detail, along with other procedures for characterizing ER of eggs in unfixed, cell-free conditions.

METHODS

Arbacia punctulata and *Strongylocentrotus droebachiensis* were obtained at the MBL, *Arbacia lixula* and *Paracentrotus lividus* at the Station Marine in Villefranche, and *Lytechinus pictus* and *Strongylocentrotus purpuratus* from Marinus, Inc. (Venice, CA). Eggs were dejellied for 1.5 min in EMC, a divalent ion-free seawater (Detering *et al.*, 1977), and then applied in seawater for 1 min to coverslips treated with polylysine (Sigma Chemical Co., St. Louis, MO; 1.5 mg/ml). Excess fluid was removed, followed by three quick washes with intracellular buffer and then application of shear. Intracellular buffer was: 350 m*M* potassium glutamate, 350 m*M* glycine, 20 m*M* Hepes, 3 m*M* $MgCl_2$, 0.57 m*M* $CaCl_2$, 10 m*M* EGTA, pH 6.8. Potassium glutamate was replaced in experiments investigating solute effects.

DiI (either $DiIC_{16}(3)$ or $DiIC_{18}(3)$) was obtained from Molecular Probes (Eugene OR). A 7.5-μg/ml DiI "suspension" was made by diluting 3 μl stock solution (2.5 mg/ml in ethanol protected from light at room temperature) into 1 ml of intracellular buffer. After mixing, the suspension was used within a few minutes (DiI aggregates collide with each other to form fewer and larger aggregates; with older suspensions, fewer cortical compartments were labeled but more intensely). Cortices were stained for 1 min, washed, and then mounted in intracellular buffer in chambers made from silicon rubber (Terasaki *et al.*, 1984). DiI sometimes stained the back of the coverslip; the resulting fluorescence was reduced by wiping with ethanol. $DiOC_6(3)$ (Terasaki *et al.*, 1984) was used for staining at 2.5 μg/ml in intracellular buffer for 10 sec.

For staining RNA, cortices were mounted in 1 μg/ml thiazole orange (Lee *et al.*, 1986; Molecular Probes; stored at 1 mg/ml in DMSO) in intracellular buffer. For Hoechst dye staining, cortices were put into pH 2 solution (350 m*M* potassium acetate, 13 m*M* magnesium chloride, adjusted to pH 2 with 5 *N* HCl) for 1 min, then into 1 μg/ml Hoechst 33258 (Hilwig and Gropp, 1975; Sigma Chemical Co.; kept at 1 mg/ml in H_2O) for 2 min, and then washed and mounted in pH 2 solution.

Immunofluorescence labeling of the calsequestrin-

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like protein has been described (Henson *et al.*, 1989). For double-labeling, cortices were stained with DiI, photographed, processed for immunofluorescence, and then rephotographed. A Zeiss IM 35 microscope was used with a Zeiss 100× plan N.A. 1.25 lens or a Nikon 100× planapo N.A. 1.4 lens and a 100-W mercury arc source. TMAX 400 or TMAX 3200 35-mm film (Eastman-Kodak, Rochester, NY) was used for photography.

RESULTS

DiI has a fluorescent head group with two long hydrocarbon chains. The hydrocarbon chains intercalate into membrane bilayers; once in a bilayer leaflet, DiI remains in the leaflet but diffuses freely within that leaflet (Honig and Hume 1986). When DiI was diluted from an ethanolic solution into aqueous buffer, it formed a suspension of microscopic aggregates. Aggregates in brownian motion were visible by fluorescence microscopy, and centrifugation (10,000*g*, 10 min) concentrated the dye at the top of the tube. We did not determine whether the aggregates were precipitates, crystals, or micelles. DiI suspensions stain membranes by random collisions of the DiI aggregates. DiI spread in the ER of egg cortices from smaller brightly labeled areas (Fig. 1); some cortical granules were also stained and the plasma membrane of some cortices was stained. Although DiI suspensions do not stain ER membranes specifically, the ER is stained well because the widespread ER membranes can be hit in many places and because the continuity of the ER allows dye spreading. $DiOC_6(3)$ (Terasaki *et al.*, 1984) was not useful because it stained all of the cortical granules as well as the ER.

Double-labeling with DiI and immunofluorescence with an antibody to a calsequestrin-like protein (Henson *et al.*, 1989) showed that labeling by the two methods was essentially identical (Fig. 2).

Thiazole orange, a dye which stains RNA, stained a network similar to that stained by DiI (Fig. 3). Mg-free buffer, which detaches ribosomes from microsomes (Sabatini *et al.*, 1966), eliminated the staining within 2 min without eliminating stainability by DiI. Hoechst dye 33258 in pH 2 solution stained a punctate network on the cortices; the discontinuity may be due to disruption by the acidic conditions.

The ER often encircles individual cortical granules on *A. punctulata* (Fig. 4), *L. pictus*, and *A. lixula* cortices. The ER is denser on cortices of *S. purpuratus* and *S. droebachiensis*, surrounding almost every cortical granule. In all species, essentially every "mesh opening" of the network surrounds at least one cortical granule.

ER was often found outside of the cortices on the "leeward" side relative to the shearing force, suggesting that cytoplasmic components had been thrown out onto the coverslip. DiI, $DiOC_6(3)$, the antibody to calsequestrin-like protein (Fig. 5), thiazole orange, and Hoechst dye 33258 all labeled these membranes.

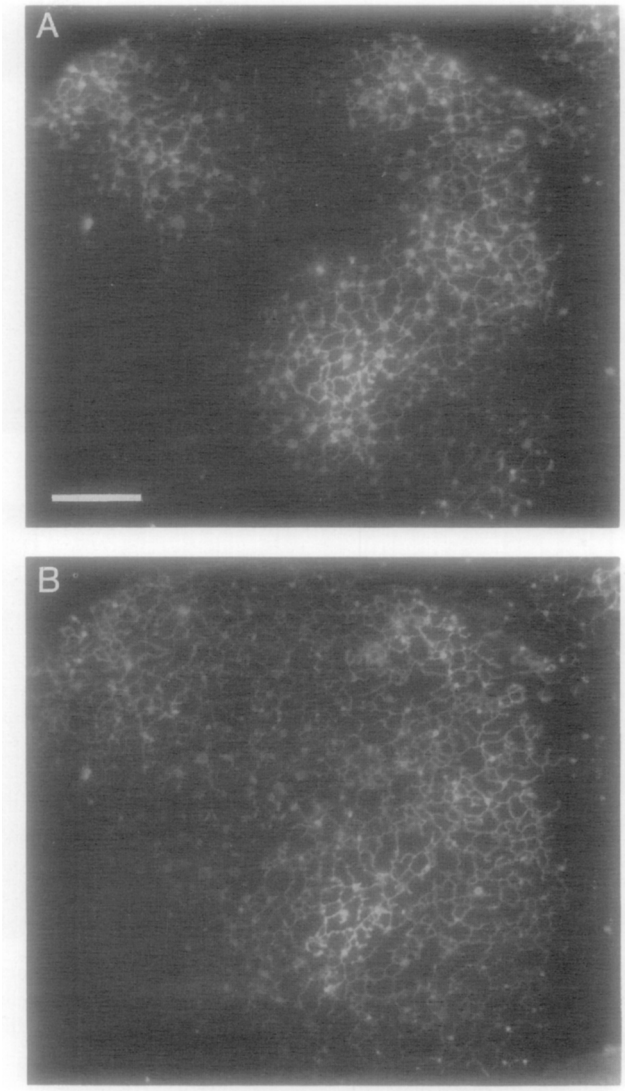

FIG. 1. Staining of cortical ER by DiI. (A) One minute after staining for 10 sec with a DiI suspension and (B) 4 min after staining. Spreading from localized regions of bright staining is consistent with random collisions by DiI aggregates with the ER. Bar = 10 μm.

Hypotonicity (1:3 dilution with water, but not 1:1) disrupted the ER. Continuity was preserved when the main solute in the buffer was potassium glutamate, potassium aspartate, sodium glutamate, mannitol, or sucrose and was also preserved in pH from 6.7 to 7.4. High chloride concentrations (350 m*M*) disrupted the ER; both cortical and extracortical ER were disrupted in potassium chloride and sodium chloride-based buffers. This may be why buffers containing high chloride sometimes fail to preserve properties of intact cells (Kostyuk *et al.*, 1976) and why early structural studies failed to

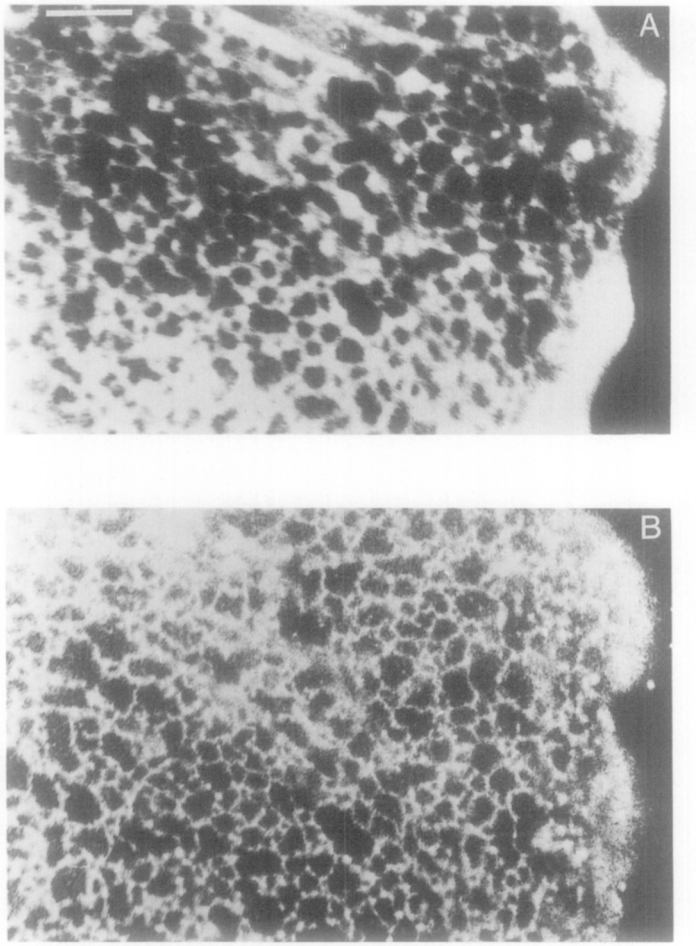

FIG. 2. Comparison with the distribution of a calcium binding protein isolated from sea urchin egg microsomes. (A) A cortex labeled with DiI. (B) The same cortex labeled with anti-calsequestrin-like protein. The patterns are essentially identical. Bar = 10 μm.

detect ER on cortices (Vacquier, 1975; Steinhardt *et al.*, 1977).

DISCUSSION

DiI rapidly stains unfixed cortical ER, producing bright images. Staining is by a "random hit" mechanism in which dye aggregates collide with and then spread in the ER.

A protein isolated from microsomes of sea urchin eggs is similar to calsequestrin, a protein that is thought to store calcium in the sarcoplasmic reticulum (Oberdorf *et al.*, 1988; Henson *et al.*, 1989). Double-labeling with an antibody to the calsequestrin-like protein and DiI produced essentially identical patterns. The distribution of this protein suggests that all parts of the cortical ER have similar calcium storage properties.

The RNA staining dyes thiazole orange and Hoechst 33258 stained the cortical ER, confirming electron microscopic evidence that the cortical ER is rough ER (Sardet, 1984). Ribosomes are bound to the ER via an N-terminal amino acid leader sequence (Walter and Blobel, 1981); presumably, cortical ER ribosomes were translating proteins at the time of isolation or were in a state of translational arrest.

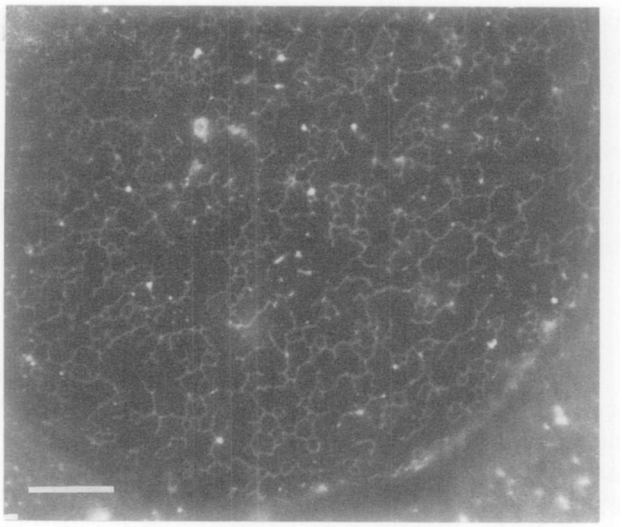

FIG. 3. Labeling with thiazole orange, a dye which stains RNA. This staining is eliminated by low Mg which detaches ribosomes. These results confirm electron microscopic evidence that the cortical ER is rough ER. Bar = 10 μm.

A calcium wave (Gilkey *et al.*, 1978), derived from internal stores (Steinhardt *et al.*, 1977), triggers cortical

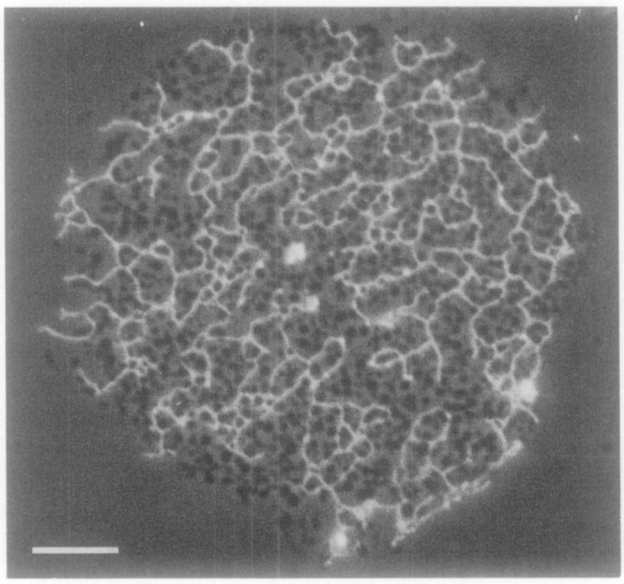

FIG. 4. A DiI-labeled cortex visualized simultaneously by phase-contrast and fluorescence. Cortical granules are often encircled by the ER. Bar = 10 μm.

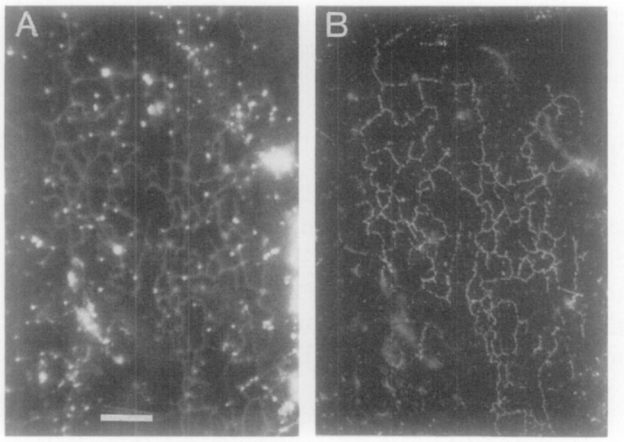

FIG. 5. Labeling of ER outside of the cortices. (A) Labeling by $DiOC_6(3)$. (B) The same field labeled by the anti-calsequestrin-like protein; the network patterns are identical. Bar = 10 μm.

granule exocytosis (Vacquier, 1975). In frog eggs, cortical granules are partially surrounded by cisternae interconnected by ER tubules; it was therefore proposed that the ER is positioned to deliver the calcium ions which initiate fusion and that propagation of the calcium wave by a network ensures coordinated fusion (Charbonneau and Grey, 1984; Campanella *et al.*, 1984). The relationship of ER and cortical granules in sea urchin eggs is consistent with these proposals.

The cortex preparation should be useful for further investigations of ER function; recent studies have demonstrated InsP3 induced release from the cortical ER (Terasaki and Sardet, 1991).

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