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Isolated Cortical Granules: A Model System for Studying Membrane Fusion and Calcium-Mediated Exocytosis

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Cortical granules are secretory vesicles bound to the inner surface of the plasma membrane of sea urchin eggs. Intact granules can be isolated by shearing away the cytoplasm of eggs which have been bonded to a protamine-coated surface. When Ca^{2+} is added to preparations of isolated granules the granules fuse with each other and release their contents. It is believed that isolated cortical granules may be an excellent model system for the biochemical study of exocytosis.

Key words: cortical granules, membrane fusion, calcium-mediated exocytosis

INTRODUCTION

Exocytosis of secretory vesicles in response to increased Ca^{2+} appears to be a general phenomenon. The exocytotic event involves the fusion of the secretory vesicle membrane with the cell membrane which results in the extracellular release of the vesicle contents. Experimental approaches aimed at understanding the mechanism of exocytosis center on at least two basic questions. First, how does Ca^{2+} trigger the process and second, what are the steps in the membrane fusion reaction?

Biochemical studies of membrane fusion and exocytosis have been hampered for lack of an advantageous model system of study (Reference 1, p. 454). The two major problems are lack of quantity and lack of synchrony of the individual exocytotic events. Some of these systems currently being studied include chemical-mediated cell fusion (2), virus-induced cell fusion (3), mast cell secretory granules (4), pancreatic zymogen granules (5), mucocysts of protozoa (6), synaptosomes of neurons (7, 8), parotid secretory vesicles (9), and bovine chromaffin granules (10).

Cortical granules are Golgi-produced exocytotic vesicles found in ova of many animal species (11). As sea urchin oocytes mature, the cortical granules migrate to the periphery of the cell and attach to the inner surface of the plasma membrane (12). Sea urchin egg cortical granules are large in comparison to other secretory vesicles (Reference 1, p. 424). In Strongylocentrotus purpuratus they average 1.3 μm in diameter and comprise 6.4% of the cell volume (13). When eggs are fertilized, the cortical granules fuse with the plasma membrane of the cell and explosively release their contents. It has been shown by use of the ionophore A-23187 (14) and by direct observation (13), that cortical granule exocytosis is triggered by Ca^{2+}. Sea urchin cortical granules contain proteases (15–17) and structural proteins (18, 19) which function in establishing the block against

polyspermy and in later morphogenesis. This paper and one which preceded it (13) present the isolated, intact cortical granule as a most advantageous model system for investigating Ca^{2+} triggering of exocytosis and the fusion of membranes of secretory vesicles.

MATERIALS AND METHODS

The method for isolating intact cortical granules has been previously presented (13). Briefly restated, the jelly coats of sea urchin eggs are dissolved by a 2-min exposure to pH 5 seawater. The eggs are washed by settling at 1 × g, aspiration of the supernatant, and resuspension in fresh seawater. A 10% vol/vol suspension of eggs is poured into a plastic culture dish which has been previously exposed to a 1% solution of protamine sulfate. The proteinaceous vitelline layer (100 Å diam, [11, 20]) covering the outer surface of the plasma membrane adheres tenaciously to the polycation-coated dish, the eggs flatten and become hemispherical. The seawater is exchanged for a Ca^{2+}-free medium containing 2–10 mM EDTA or EGTA, pH 8. Any isosmotic (1 osmol, pH 5–8.5) medium lacking Ca^{2+} and containing either chelator can be used. After 2-min in this medium, a jet of the same medium is directed at the adhering cells. The shear force ruptures the cells and washes away the cytoplasm. The dish can be tilted on edge and all contaminating cytoplasm washed away. The portions of vitelline layer which had adhered to the dish remain bound to the dish and the cortical granules remain bound to the plasma membrane-vitelline layer complex. It is not known how much of the plasma membrane remains bound to the vitelline layer. It is possible that the plasma membrane remains only in those areas where its outer surface is bonded to the vitelline layer and its inner surface to the cortical granule (Fig. 4). Dishes coated with "lawns" of intact cortical granules can be prepared in pure form in a matter of seconds (13). The jet of isolation medium can be directed from a 500-ml hand-operated laboratory squirt bottle.

For scanning electron microscopy the preparations were fixed for 2 hr in 2% glutaraldehyde in isolation medium, dehydrated in ethanol and amyl acetate, critical point dried in CO_2, and coated with gold. For transmission electron microscopy the material was fixed for 2 hr in 2% glutaraldehyde followed by 20 min in 1% OsO_4. After dehydration in ethanol the dish was flooded with 100% Epon which was changed twice before polymerization. After polymerization, broken fragments of the plastic dishes were sectioned and the sections stained with lead citrate.

RESULTS

Isolated Cortical Granules

What remains of egg cells after shearing away their cytoplasm is shown in Figs. 1, 2, and 4. The edge of an adhering vitelline layer appears on the left in Fig. 2. The vitelline layer in this species has regularly spaced, noblike projections (21) which are clearly visible in this micrograph. The individual cortical granules are firmly attached to this layer. The granule layer was irrigated with a very strong jet of isolation medium and bare patches where granules have been washed away are seen (Fig. 2). The density of the cortical granules can be as high as 74/100 μm^2. Figure 1 shows a group of intact granules at higher magnification. Some evidence for the possible interconnection of adjacent granules with strandlike material is apparent (13). Isolated, intact granules are presented in thin section in Fig. 4. This section, through a fragment of plastic dish, shows the granules bound to a layer that is believed to be a composite of the plasma membrane and vitelline layer.

Ca^{2+} -Mediated Fusion of Granule Membranes and the Release of Granule Contents

If the isolation medium containing chelator is removed and medium containing Ca^{2+} added (> 0.2 mM Ca^{2+}) the cortical granules instantaneously fuse with each other and release their contents (13). Such a fused mass of cortical granule membranes fixed in glutaraldehyde 5 sec after the addition of Ca^{2+} is shown in Fig. 3. The internal contents

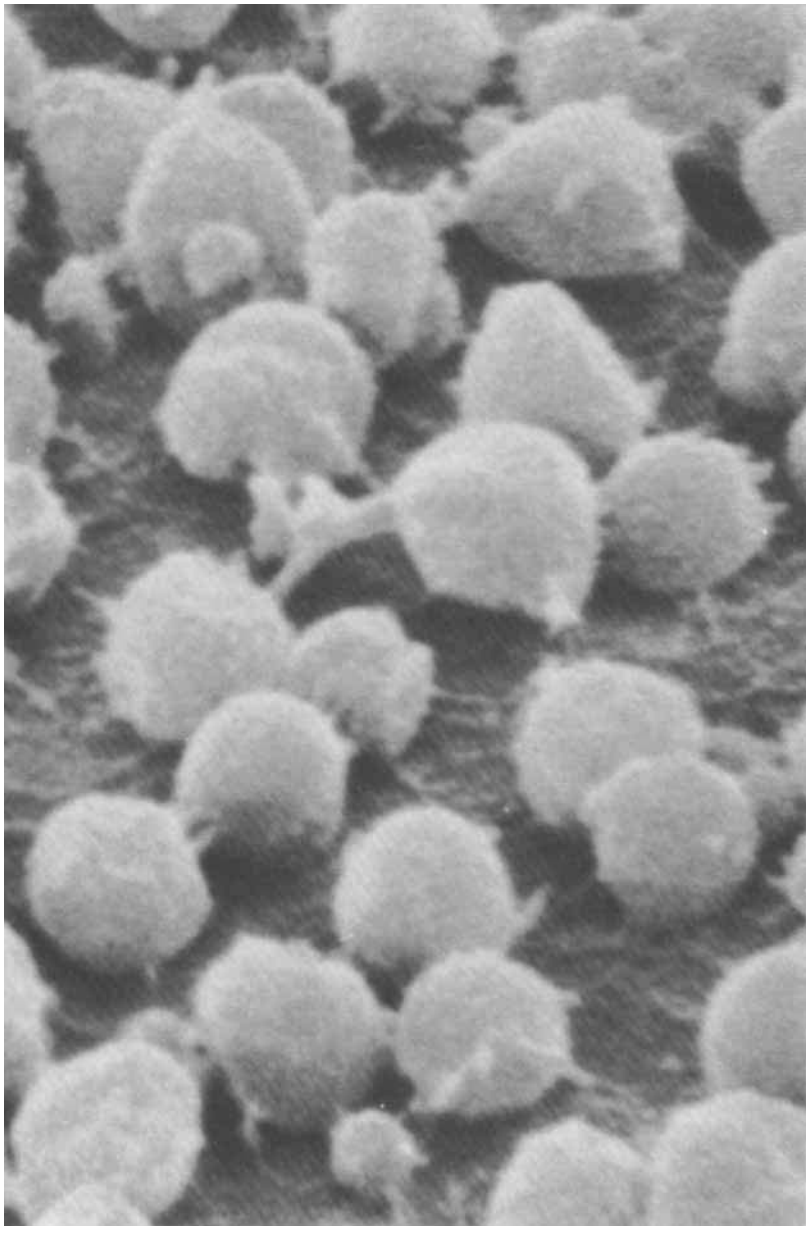


Fig. 1. SEM of isolated cortical granules bound to the inner surface of the plasma membrane, × 22,240 (reproduced with permission of Academic Press).

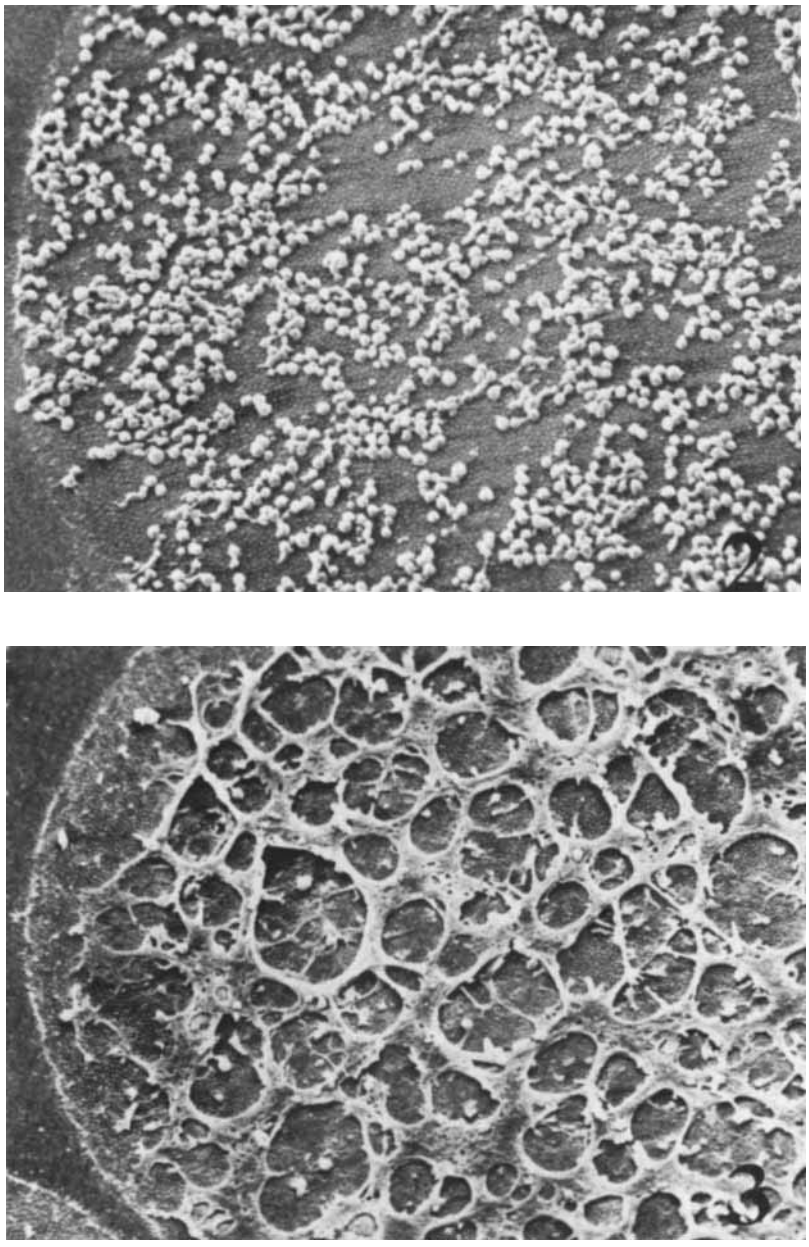


Fig. 2. SEM of the remains of one sea urchin egg after the cytoplasm is sheared away with a jet of isolation medium, × 1,825.

Fig. 3. Ca^{2+}-fused cortical granules fixed in glutaraldehyde 5 sec after Ca^{2+} addition, × 1,775.

of the cortical granules are released upon fusion. This can be determined by phase microscope observation of the condensing paracrystalline material that comprises the major structural protein of the fertilization envelope (19), and biochemically by the presence of β-1,3-glucanase in the surrounding medium (22). This enzyme is a marker for cortical granules, its function in fertilization remains unknown (17). Thin sections (Fig. 5) of Ca^{2+}-fused cortical granules provide evidence that actual fusion of granule membranes has occurred. This in vitro system, which requires only seconds to prepare, allows one instantaneously to evoke secretory granule membrane fusion in quantity. It is very easy to prepare circular "lawns" of granules 2 cm in diameter (13). Adjusting for bare spaces between cell remains in the lawn, one may expect a cortical granule density as great as 50/100 μm^2. A circular lawn of 2 cm diameter could thus contain 1.57×10^8 individual cortical granules. Each granule is bounded by 5.3 μm^2 of membrane. Such a lawn could therefore contain 8.3 cm^2 of secretory granule membrane.

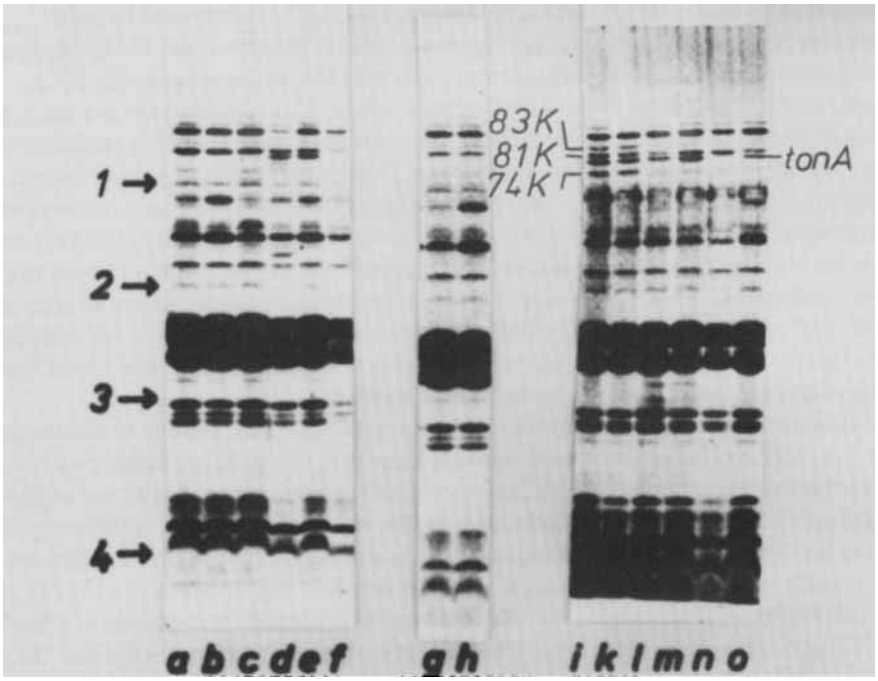
Granule to granule fusion appears to be more favored than granule to plasma membrane fusion (Fig. 5). For this reason one can argue that this in vitro system may not represent a true exocytosis. The artificial nature of the preparation, in that no supporting cortical cytoplasm surrounds the isolated granules, and the electrostatic adhesion of the vitelline layer-plasma membrane composite to the dish might be conditions which would favor granule to granule fusion. Also, as the granules swell in response to Ca^{2+} (13), their equatorial surfaces (Fig. 4) would be the points of first contact, and granule to granule fusion may be favored at these sites. The great advantages of this system are that the preparations are clean, they are bound to a supporting surface, they are prepared in seconds at minimal expense, and they fuse with each other and release their contents in response to Ca^{2+}.

Other Properties of Isolated Cortical Granules

As is true with other secretory vesicles, Ba^{2+} and Sr^{2+} will substitute for Ca^{2+} in mediating cortical granule release, but Mg^{2+} will not (13). The cortical granules will respond to Ca^{2+} at pHs ranging from 4.5 to 8.5. Exposure to pH 9.0 for 30 min, followed by readjustment to pH 8.0, destroys the "Ca^{2+}-trigger" and the granules fail to release when Ca^{2+} is added. Isolated cortical granules are sensitive to hypotonicity and begin lysing at osmolarities below 0.75 osmol. Hypertonicity does not seem to damage the "Ca^{2+}-trigger" because exposure to isolation medium 4 osmol in tonicity for 30 min followed by return to 1 osmol does not impair Ca^{2+}-mediated granule fusion. Isolated granule preparations are extremely stable and can be kept in isolation medium for 3 days at 22°C and still respond instantly to Ca^{2+} addition. As previously reported, cortical granule release is not affected by a 2-hr exposure to 0.1 mg/ml colchicine, colcemid, and 0.05 mg/ml cytochalasin B (13). The Ca^{2+}-mediated granule release is reversibly inhibited by local anesthetics such as procaine. It is irreversibly inhibited by exposure to La^{3+}, Co^{2+}, and Mn^{2+} (13).

Ca^{2+} Concentration and Cortical Granule Fusion

Several workers have measured the intracellular concentration of Ca^{2+} in sea urchin eggs before and after fertilization (Table I). Early work showed by chemical analysis that the total amount of intracellular calcium does not change after fertilization. The amount of dialyzable calcium, however, increases threefold (to 1.5 mM) after fertilization. These



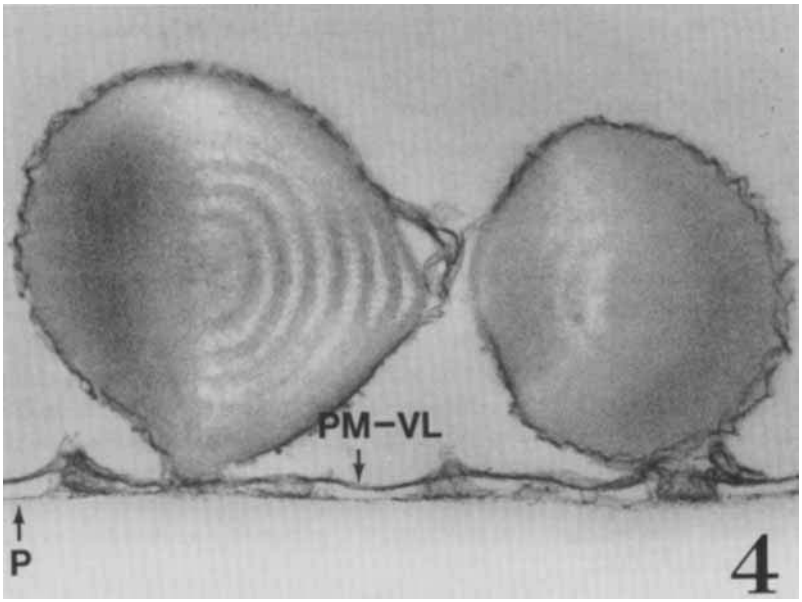


Fig. 4. TEM thin section of isolated cortical granules bound to the surface of the plasma membrane-vitelline layer composite which is itself electrostatically bonded to a plastic culture dish. P, plastic surface, PM-VL, plasma membrane-vitelline layer composite, × 50,400 (reproduced with permission of Academic Press).

Fig. 5. TEM thin section of Ca^{2+}-fused membranes of isolated cortical granules, × 52,400.

TABLE I. Determination of "Free Ca $^{2+}$" in Sea Urchin Eggs

	Unfertilized	Fertilized	Reference
1	0.5 mM	1.5 mM	(24)
2	0.4 mM	–	(25)
3	0.1 mM	1.0 mM	(26)
4	0.26 mM	–	(14)

data were interpreted by presenting the hypothesis that "free" Ca^{2+} is released at fertilization from a "bound" form such as a Ca-proteinate (24). More recently, by use of isotope exchange data, the "free" (ionic) Ca^{2+} of unfertilized eggs was found to be 0.4 mM (25). From the data of Steinhardt and Epel (14), who homogenized eggs in 0.55 M KCl, the Ca^{2+} remaining soluble after centrifugation at 37,000 X g can be computed to be 0.26 mM. Other workers, using the more precise technique of equilibrium dialysis, have calculated the unfertilized egg to be 0.1 mM ionic Ca^{2+} and the fertilized egg 1.0 mM (26). In their experiments a sharp increase in the dissociation constant of the unfertilized egg homogenate was observed after the addition of Ca^{2+} to a concentration of 0.3 mM (26).

The following experiment was performed to determine the lowest Ca^{2+} molarity which would evoke instantaneous fusion of isolated cortical granules. Lawns of cortical granules were prepared in clear plastic dishes (13). When viewed against a black background with the unaided eye a lawn appears as a gray film on the surface of the dish. It is important to keep the granule lawn completely submerged in isolation medium because the granules fuse if contact with the air-liquid interface occurs. When the granules fuse the gray film disappears and the surface of the dish appears clear. Medium containing various concentrations of Ca^{2+} was added to dishes containing granule lawns. Within 5 min of isolation (22°C), 0.05 mM Ca^{2+} (added to a solution containing 0.5 M NaCl, 0.02 M $MgCl_2$, 0.001 M Tris, pH 7.5) would not provoke the granule fusion reaction. Limited patches of granule fusion, seen as clear areas in the gray lawn of granules, were produced in 0.1 mM Ca^{2+}. A concentration of 0.2 mM Ca^{2+} or greater was necessary to achieve instantaneous clearing of the granule lawn. As previously mentioned, in the intact egg cell the Ca^{2+} which elicits cortical granule exocytosis supposedly comes from an intracellular store of "bound" calcium (14, 24). Our experiments with isolated cortical granule layers show that the "free" Ca^{2+} of egg cytosol may have to increase to a minimum of 0.2 mM before granule exocytosis is initiated.

DISCUSSION

The technique of sticking cells to surfaces coated with polybases has been used with many types of cells (27–33). The additional procedure of shearing away the cytoplasm of the stuck-down cell has allowed observation of the inner surface of the cell membrane. In addition to the use of this technique to study cortical granules (13), some intriguing results have been reported recently on other types of cells. Clarke et al. (34) have shown actin filaments associated with the inner surface of the cell membrane of Dictyostelium amebas. Boyles and Bainton (35) have observed the formation of KI-soluble filaments associated with the plasma membrane of polymorphonuclear leukocytes during active phagocytosis of yeast. Because there is evidence that microtubules and microfilaments may

anchor lymphocyte surface receptors for Con A (36–38), it would be of great interest to apply this technique to lymphocytes to determine if patching and capping of Con A receptors involved a redistribution of microtubules and microfilaments on the inner surface of the lymphocyte membrane.

Although cortical granules share the Ca^{2+} reaction general to all secretory vesicles (Ref. 1, p. 441), they do exhibit significant differences. Exocytosis of many types of secretory granules may require the action of a protease (39). Ca^{2+}-mediated fusion of isolated cortical granules is not inhibited by high concentrations and long exposure times to several protease inhibitors (13). Microtubules (40) and tubulin (41, 42) have been shown to be associated with secretory vesicles, and exocytosis in other systems is inhibited by colchicine, colcemid, and cytochalasin B (reviewed in Ref. 43). Isolated cortical granules exposed to these drugs show no inhibition of the Ca^{2+} fusion reaction (13). At this time we do not know if tubulin or any loosely associated proteins (44) play roles in cortical granule exocytosis.

Actin and myosin appear to be associated with the membrane of chromaffin granules (45). Although these proteins have not as yet been demonstrated in isolated cortical granules, sea urchin eggs contain substantial amounts of both (46, 47). In this context, it is interesting to note that isolated cortical granules will detach from the vitelline layer-plasma membrane composite when exposed for 20 min to 90% 0.5 M KI, 10% isolation medium.

The location of the store of intracellular "bound Ca^{2+}" which releases Ca^{2+} active in cortical granule exocytosis is unknown at this time. Evidence that the store may be associated with the granule membrane has been presented (13). This evidence consists of the demonstration that a wave of granule fusion will propagate in one direction across a lawn of isolated granules and also that the local anesthetic procaine hydrochloride will reversibly inhibit Ca^{2+}-mediated granule fusion (13). Location of the bound Ca^{2+} store on the cortical granule membrane is not an unreasonable idea. The highest concentration of calcium in zymogen granules is found on the granule membranes (48). This would mean that the cortical granule membrane would contain the Ca^{2+}-triggering mechanism and the "bound" calcium store. Quantitation of Ca^{2+} binding to secretory granule membrane has so far been studied only in chromaffin granules (49). No dramatic localization of calcium such as that found in platelets (50) and glial cells (51) has been found in echinoderm eggs. Cortical granules may represent "stripped down" exocytotic vesicles in which microtubules, microfilaments, and protease action are not required for discharge. Thus, the reactions initiated by Ca^{2+} binding to the membranes of isolated granules which lead to fusion of granule membranes may be more accessible to study in this system. Bach (39) has proposed that Ca^{2+} may activate a phospholipase A in the secretory granule membrane. If so, one may expect to find lysophospholipids in the medium surrounding fusing cortical granules.

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