Isolation and Characterization of Sea Urchin Egg Cortical Granules

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ABSTRACT A method has been developed to isolate cortical granules (CG) free in suspension. It involves the mechanical disruption of the CG from CG lawns (CGL; *Dev. Biol.* 43:62–74, 1975) and concentration of the CG by low speed centrifugation. The isolated CG are intact and are a relatively pure population as judged by electron microscopy. Granule integrity is confirmed by the fact that isolated intact CG are radioiodinated to only 0.05% of the specific activity of hypotonically lysed CG. Purity of the CG preparation is assessed by the enrichment (four- to sevenfold) of CG marker enzymes and the absence or low activity of plasma membrane, mitochondrial, cytoplasmic, and yolk platelet marker enzyme activities. CG isolated from ^{125}I-surface-labeled eggs have a very low specific radioactivity, demonstrating that CG contamination by the plasma membrane-vitelline layer (PM-VL) is minimal. CG yield is ~1% of the starting egg protein. The CG isolation method is simple and rapid, 4 mg of CG protein being obtained in 1 h. Isolated CG and PM-VL display distinct electrophoretic patterns on SDS gels. Actin is localized to the PM-VL, and all bands present in the CGL are accounted for in the CG and PM-VL. Calmodulin is associated with the CGL, CG, and PM-VL fractions, but is not specifically enriched in these fractions as compared with whole egg homogenates. This method of isolating intact CG from unfertilized sea urchin eggs may be useful for exploring the mechanism of Ca^{2+}-mediated CG exocytosis.

The exocytosis of secretory organelles in response to elevated cytosolic Ca^{2+} is a general phenomenon of eukaryotic cells. Although the ultrastructural details of exocytosis are known in a variety of cells (12, 34, 49, 62), little is known about the molecular mechanism by which Ca^{2+} triggers the fusion of the secretory organelle membrane with the plasma membrane (10).

The eggs of many animals contain membrane-limited, Golgi-derived secretory organelles known as cortical granules (CG), which lie directly subjacent to the plasma membrane. In eggs of the sea urchin *Strongylocentrotus purpuratus* the CG are ~1 μm in diameter, comprise 6.4% of the total egg volume, and are closely associated with the plasma membrane (80). Approximately 25–30 s postinsemination, a wave of CG exocytosis begins to sweep over the egg surface from the point of sperm fusion. The discharge of all CG requires 25–30 s and is, no doubt, one of the most massive and synchronous exocytotic responses known for any cell. The wave of CG exocytosis results from the release of Ca^{2+} from unknown intracellular stores (11, 29, 74, 75, 87). The enzymes and structural proteins which are released extracellularly by the CG function in the elevation of the vitelline layer and its transformation into the fertilization envelope, in the formation of the hyaline layer necessary for morphogenesis, and in the establishment of the complete block to polyspermy (65). Because of the massive amounts of sea urchin eggs that are easily obtained, and since this exocytosis is extremely synchronous, the sea urchin egg CG is an advantageous system for the biochemical analysis of exocytosis.

Several methods have been developed to isolate CG from sea urchin eggs. Homogenization of eggs and subsequent zonal centrifugation yields large quantities of fragmented CG which may, in the future, prove valuable for the isolation of CG proteins involved in the Ca^{2+} triggering mechanism (66, 68). However, at this time, the usefulness of this method has been hampered by the large quantities of starting material needed and contamination of the CG by other cellular components. Homogenization of unfertilized eggs in isosmotic media containing Ca^{2+} chelators yields isolated fragments of the egg

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cortex composed of the vitelline layer and the plasma membrane with its attached intact CG (23, 64, 83). When Ca^{2+} is added to these fragments, the CG fuse with each other and with the plasma membrane in what in some cases appears, ultrastructurally, to be a true exocytotic reaction (20). However, such isolation techniques are not amenable to the biochemical analysis of the CG themselves.

We have developed a simple method to isolate fragments of the egg cortex which are bound to plastic culture dishes. These preparations, known as cortical granule lawns (CGL; 80), have been used in studies of the Ca^{2+}-mediated CG discharge reaction (2, 3, 73, 75). The CGL are virtually free of cytoplasmic contamination and are prepared by a single step procedure requiring only seconds. Here we describe a method to isolate milligram quantities of CG free in suspension. The isolated CG are morphologically intact and are a homogeneous population, as judged by several criteria.

MATERIALS AND METHODS

Materials

Reagents and suppliers were: Na ^{125}I (carrier free) and [^{3}H]ATP (Amersham-Searle Arlington Heights, IL; or ICN, Cleveland, OH); N-α-(*p*-tosyl)-L-arginine [^{3}H] methyl ester (TAME: Amersham-Searle); unlabeled nucleotides, cyclic nucleotides, and laminarin (United States Biochemical Corp., Cleveland, OH); enzyme substrates (Sigma Chemical Co., St. Louis, MO); Dowex AG50W-X8 (100–200 mesh) and neutral alumina (AG7, 100–200 mesh) (Bio-Rad Laboratories, Richmond, CA); DNase I-Sepharose (Worthington Biochemicals Freehold, NJ); Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). Fluphenazine·2HCL was a gift from E. R. Squibb and Sons, Inc. (Princeton, NJ) and *Dictyostelium discoideum* actin was kindly provided by Dr. J. A. Spudich, Stanford University, Stanford, CA (71).

Methods

COLLECTION AND PREPARATION OF EGGS: Eggs of the sea urchin, *Strongylocentrotus purpuratus*, were spawned into sea water by pouring 0.5 M KCl into the body cavities. Egg jelly coats were removed by adjusting the pH to 5 with 0.1 N HCl. After 4 min the pH was readjusted to 8 by the dropwise addition of 1 M Tris (pH 8). The eggs were then washed twice by settling in artificial sea water (ASW:454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 9.6 mM $CaCl_2$, 27.1 mM $MgSO_4$, 4.4 mM $NaHCO_3$, 10 mM Tris-HCl, pH 8.0) and resuspended to a concentration of 20% (vol/vol).

PREPARATION OF CORTICAL GRANULE LAWNS: Plastic tissue culture dishes (Falcon #3002, 60 × 15 mm; Falcon Labware, Oxnard, CA) were treated for 5 min with 2 ml of 1% aqueous solution of protamine sulfate (Sigma Grade X), rinsed with distilled water, and air dried (80). Three ml of egg suspension were added to each dish and allowed to bond for 2 min. The egg lawn was washed into Ca^{2+} free ASW (CaFASW), and then sheared by a jet of isolation medium (Buffer A) delivered from a hand-held squirt bottle. Buffer A contained 1 M glycerol, 57.3 mM $MgCl_2$, 7.68 mM $CaCl_2$, 10 mM EGTA, and 0.5 mM ATP, pH 8.0 (free Ca^{2+} was ~5×10^{-7} M). The shear force generated by the stream of buffer A washed away the egg cytoplasm, leaving a CGL bound to the dish (80).

PREPARATION OF ISOLATED CORTICAL GRANULES: Cortical granules were mechanically dislodged from the CGL by repeated squirting from a Pasteur pipet containing a buffer composed of 0.45 M KCl, 0.4 M sodium isethionate, 0.5 mM ATP, 2.35 mM $MgCl_2$, 5.31 mM $CaCl_2$, 10 mM EGTA, and 10 mM PIPES, pH 7.0 (Buffer B; free Ca^{2+} was ~5×10^{-7} M). All procedures were performed at 22°C and the isolated CG suspension was stored at 4°C.

ELECTRON MICROSCOPY: Isolated CG were concentrated on a discontinuous sucrose density gradient composed of 5 ml each of 30% and 60% sucrose made in buffer B containing 1% bovine serum albumin (BSA). The CG banded at the 30–60% interface after centrifugation in a HB-4 (Sorvall DuPont, Newtown, CT) rotor at 2,600 *g* (10 min; 4°C). The CG were removed with a Pasteur pipet, an equal volume of fixative (3% glutaraldehyde, 1% paraformaldehyde, 0.26 M sodium acetate, pH 6.0) was added, and the material fixed overnight (5°C). The fixed CG, enmeshed in the precipitate formed by the cross-linking of BSA, were then concentrated by centrifugation (2,600 *g*; 10 min). The pellet was gently dispersed with a spatula and washed overnight in excess 0.5 M sodium acetate, pH 6.0. The CG were then postfixed in 1% OsO_4, 0.5 M sodium acetate (pH 6.0) for 30 min, passed through an ethanol-propylene oxide dehydration series and embedded in Epon.

PREPARATION OF WHOLE EGGS AND CORTICAL GRANULES FOR ENZYME ASSAYS: After a low speed centrifugation in a hand centrifuge, the pelleted, dejellied eggs were resuspended in 3 vol of a buffer containing 2 mM Tris (pH 7.4), 10 mM NaCl, 10 mM KCl, and 80 μM EGTA (buffer C) and homogenized with seven passes in a Potter-Elvehjem homogenizer (0°C). The crude whole-egg homogenate was then used fresh or frozen in aliquots at −70°C.

The isolated CG suspension (in buffer B) was centrifuged for 10 min at 2,600 *g* in a HB-4 rotor (4°C). The CG pellet was resuspended in buffer C to the appropriate protein concentration. Addition of this hypotonic buffer caused the CG to lyse, which was detected by a clearing of the opaque pellet. In some experiments buffer C was supplemented with 10 mM benzamidine or 5 μM phenylhydrazine to inhibit CG-associated proteoesterase and ovoperoxidase (28) activity.

ENZYME ASSAYS: Product formation was linear with respect to time and protein concentration. Conditions for optimal enzyme activity were established for each enzyme.

Acid phosphatase (pH 5.0) was measured by a modified method of Shibko and Tappel (69). Whole egg homogenates or CG lysates in buffer C containing 0.1% Tergitol NPX (Sigma Chemical Co.) were incubated at 24°C in 1 ml containing 100 mM sodium acetate (pH 5) and 5 mM *p*-nitrophenyl phosphate. Reactions were terminated by the addition of phosphotungstic acid (in 0.1 N HCl) to 0.66%, and the *p*-nitrophenol liberated was determined colorimetrically (A_{410nm}) by the addition of NaOH to 0.16 N.

Adenylate cyclase activity was assayed in 250 μl of a mixture containing 30 mM tris (pH 7.9), 1 mM ATP, 5 mM $MnCl_2$, 2.2 mM cyclic AMP, 10 mM creatine phosphate, 75 μg creatine kinase, 5–6 × 10^6 cpm [^{3}H]ATP and 200 μg of protein of either egg homogenate or CG lysate. The assay was done at 23°C for 20 min, the reactions were stopped by the addition of 2 ml of 0.5 N $HClO_4$ and cyclic AMP purified (39).

For Na^+/K^+-ATPase activity, whole eggs and CG pellets were resuspended in a buffer containing 50 mM Tris (pH 7.4), 10 mM benzamidine, and 5 μM phenylhydrazine and homogenized or lysed. Assays were done in 400 μl containing 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 1 mM ATP, and 100 μg of either egg or CG protein. Na^+/K^+-ATPase activity was expressed as the ouabain inhibitable activity. Incubation was done for 45 min at 25°C; reactions were terminated by the addition of 80 μl of 5% SDS (78) and inorganic phosphate was measured (51).

For cytochrome *c* oxidase activity (70), the cytochrome *c* was reduced before assay by the addition of sodium hydrosulfite to 4 mM (15). Enzyme activity was determined at 24°C (A_{550nm}) in 1 ml containing 70 mM potassium phosphate (pH 7.0), 15 μM ferrocytochrome *c*, 0.1% Tergitol NPX and 100 μg of protein of either egg homogenate or CG lysate in buffer C containing 10 mM benzamidine and 5 μM phenylhydrazine. Assays were done for 3–5 min, and maximal cytochrome *c* oxidation was determined by the dropwise addition of a saturated solution of $K_3Fe(CN)_6$. Data are expressed as the first-order rate constant according to the equation of Smith (70).

For exo-$\beta(1 \rightarrow 3)$-glucanohydrolase (glucanase) activity (77), egg homogenate and CG lysate were prepared in buffer C containing 10 mM benzamidine and 5 μM phenylhydrazine.

Glucose-6-phosphate dehydrogenase was assayed spectrophotometrically (85) in a reaction coupled to the generation of NADPH. The reaction mixture (1 ml) contained 40 mM Tris (pH 7.8), 4 mM $MgCl_2$, 0.2 mM NADP, 3.34 mM glucose-6-phosphate, and 100 μg of either egg or CG protein. Reactions were done for 4–5 min (24°C) after an initial 8-min equilibration in the absence of the enzyme. Enzyme activity was expressed as nanomoles of NADP reduced/min/mg protein, using a molar absorption coefficient ($\sum_{340nm}$) of 6.22×10^3 M^{-1} cm^{-1} for NADPH.

For 5′-nucleotidase, incubation mixtures consisted of 50 mM Tris (pH 8), 1 mM EDTA, 5 mM 5′-AMP, and 200 μg of egg or CG protein in buffer C containing 10 mM benzamidine and 5 μM phenylhydrazine. Assays were done at 37°C for 90 min in 400 μl. Reactions were stopped and inorganic phosphate was determined (51).

For ovoperoxidase (32), incubation mixtures (1 ml) contained 10 mM Tris (pH 7.9), 550 mM NaCl, 0.005% dimethoxybenzidine, 30 μM H_2O_2, and 25–150 μg of egg or CG protein in buffer C containing 10 mM benzamidine. Enzyme activity (24°C) was determined using a molar absorption coefficient ($\sum_{460nm}$) of 1.13×10^4 M^{-1} cm^{-1} for oxidized dimethoxybenzidine (85).

Proteoesterase activity was measured (24°C) using *N*-α-(*p*-tosyl)-L-arginine [^{3}H]methyl ester ([^{3}H]TAME) as a substrate (60). The assay contained 250 mM Tris (pH 8), 0.25 mM TAME, 10^5 cpm [^{3}H]TAME, and 25–150 μg egg or CG protein in buffer C containing 5 μM phenylhydrazine.

Cyclic nucleotide phosphodiesterase activity was assayed (30°C; buffer B or C) using 1 μM cyclic GMP (84).

PAGE: Discontinuous PAGE was performed using an anionic pH 9.7 glycine system (86). SDS (0.1%) was added to the gels and reservoir buffer when electrophoresis was done under denaturing conditions. Gels were stained for protein with Coomassie Blue (R-250) or for carbohydrate with periodic acid-

Schiff reagent. Egg lawns, CGL, and isolated CG were prepared in buffers A and B containing 10 mM benzamidine and 5 μM phenylhydrazine. Plasma membrane-vitelline layer complexes (PM-VL) were prepared by dislodging the CG from the CGL and solubilizing the material that remained attached to the culture dish in 10% SDS. Whole-egg lawn protein was obtained by adding 10% SDS containing 10 mM benzamidine and 5 μM phenylhydrazine to the culture dishes. Before the addition of SDS, the CGL and PM-VL were washed once with 1 ml of ice-cold 10% TCA, followed by two washes with excess cold absolute ethanol. Isolated CG were pelleted at 2,600 *g* (10 min; 4°C) and the pellet was washed with 10% TCA and absolute ethanol before addition of 10% SDS. Immediately upon addition of SDS, the fractions were placed in a boiling water bath for 3 min. Immediately before electrophoresis, the samples were heated again for 3 min in sample buffer containing 2% mercaptoethanol and 100 μg of protein loaded per lane.

LOCALIZATION OF ENZYMATIC ACTIVITIES ON POLYACRYLAMIDE GELS: Glucanase activity was detected on nondenaturing and denaturing 5% and 10% polyacrylamide gels. Cortical granules were collected in media containing 10 mM benzamidine and 5 μM phenylhydrazine (buffers A, B, or C). For SDS gels the sample was prepared, run, and assayed for enzyme activity as published (77). For nondenaturing gels, the isolated CG were lysed in buffer C containing 10 mM benzamidine and 5 μM phenylhydrazine, resuspended in stacking gel buffer, and electrophoresed. Frozen gels were then sliced (1-mm sections) and assayed for enzyme activity (77).

Proteoesterase activity of CG lysed in buffer C containing 5 μM phenylhydrazine was measured after electrophoresis in nondenaturing gels. The 1-mm gel slices were then preincubated with 100 μl of 250 mM Tris (pH 8) for 3.5 h (24°C). An aliquot (45 μl) was then added to the incubation mixture containing the substrate ([^{3}H]TAME).

RADIOIODINATION PROCEDURES: Artificial sea water (ASW) and all isolation media contained 10 mM benzamidine and 5 μM phenylhydrazine. A 50-ml egg suspension (20% vol/vol in ASW) was transferred to an Iodogen-coated 1-liter beaker (1.25 mg Iodogen/10 ml of $CHCl_3$, evaporated with N_2) that had been rinsed three times with ASW. One mCi $Na^{125}I$ was added and the beaker swirled for 7 min (24°C). The eggs were then washed five times in ASW by hand centrifugation. The washed eggs were poured onto protamine sulfate-coated culture dishes, and the egg lawn, CGL, PM-VL, and CG were collected. The various fractions were either suspended directly in 10% SDS (egg lawn) or washed with 10% TCA and absolute ethanol before addition of SDS (CGL, PM-VL, CG). The samples were boiled for 3 min and the distribution of radioactivity in the various macromolecular species was determined.

Iodination of intact CG was accomplished by two methods. In the first, the dilute suspension of CG was concentrated on a 30–60% sucrose gradient (without BSA). The concentrated CG (1.5 ml) were then added to an Iodogen-coated 50-ml beaker containing 0.5 mCi $Na^{125}I$ which was swirled for 7 min and the reaction was stopped by decanting the CG into another beaker. The CG suspension was then diluted with buffer B, so that the final sucrose concentration was 15%. The suspension was then recentrifuged on a sucrose gradient, and the 30–60% interface was isolated, diluted again with buffer B, and pelleted by centrifugation at 2,600 *g* (10 min; 4°C). The pelleted CG were lysed in buffer C, extracted with 10% TCA, centrifuged (17,400 *g*) and the pellet was washed twice with absolute ethanol. The washed pellet was then heated (3 min) in 10% SDS.

In the second method, the dilute suspension of CG (30 ml) was immediately transferred to a 1-liter Iodogen-coated beaker containing 0.5 mCi $Na^{125}I$. After 7 min, the CG suspension was centrifuged at 2,600 *g* (10 min; 4°C). The pelleted CG were then lysed, extracted, washed, and resuspended in SDS. Radioiodination of CG after lysis in buffer C was carried out in the same manner.

Determination of the distribution of ^{125}I into macromolecules, trypsin- and pronase-sensitive sites, and lipid were carried out using previously described methods (36, 37, 43, 53).

AFFINITY CHROMATOGRAPHY: The isolation of actin from the PM-VL fraction was done by the DNase I-Sepharose method (72). After dislodging and removing the CG, the PM-VL remaining bound to the culture dishes were extracted with 0.6 M KI, 1 mM dithiothreitol, 0.2 mM $MgCl_2$, 0.1 mg/ml soybean trypsin inhibitor (SBTI), 0.2 mM ATP, 10 mM triethanolamine, pH 7.5, for 4.5 h at 24°C. The extract was then chromatographed on a 0.7 × 4-cm column of DNase I-Sepharose at 4°C (72). The various fractions were dialyzed against water, lyophilized, and electrophoresed on SDS polyacrylamide gels.

Fluphenazine-Sepharose 4B was prepared according to Charbonneau and Cormier (13). CG lysate was prepared using buffer C, and the lysate was then dialyzed against the column equilibration buffer (10 mM HEPES, pH 7, 0.5 mM $CaCl_2$) before chromatography.

PREPARATION OF CALMODULIN-DEFICENT BOVINE BRAIN CYCLIC NUCLEOTIDE PHOSPHODIESTERASE: Calmodulin-deficient phosphodiesterase was prepared from bovine brain (14). The enzyme was taken through the $(NH_4)_2SO_4$ fractionation step and then chromatographed on DEAE-cellulose. The enzyme, which eluted from the DEAE-cellulose, was activated by calmodulin. Protein determinations were done using BSA as a standard (54).

RESULTS

Parameters of the Cortical Granule Isolation Procedure

The presence of EGTA in all media is an absolute requirement because CG on CGL undergo immediate disruption when free Ca^{2+} is present in micromolar concentrations (2, 4, 75). However, isolated CG are stable (as viewed microscopically) for several hours if Ca^{2+} is included in the media buffered with EGTA so that free Ca^{2+} is ~5×10^{-7} M. Granule stability is also enhanced by the addition of sodium isethionate to buffer B.

Cortical Granule Yield

Table I presents the approximate yield of CG protein. The CGL represents ~2.9% of the starting egg protein. Each culture dish yields ~130 μg of isolated CG protein and 23 μg of PM-VL. The difference in the CGL protein as compared with the isolated CG pellet and PM-VL fraction can be recovered in the supernatant above the pelleted CG. Approximately 4 mg of CG protein can be obtained in 1 h.

Morphology of Isolated Cortical Granules

Cortical granules in buffer B appear spherical and intact when viewed by oil immersion phase contrast microscopy (Fig. 1). Electron microscopy (Fig. 2) shows that the CG are com-

TABLE I

Recovery of Protein During Cortical Granule Isolation

Fraction	mg Protein/dish
Egg lawn	14.27 ± 1.74 (5)*
Cortical granule lawn (CGL)	0.42 ± 0.16 (7)
Plasma membrane-Vitelline layer fraction (PM-VL)	0.023 ± 0.005 (4)
Isolated cortical granules (CG)	0.132 ± 0.028 (15)

* Data represent the mean ± SD. (n) = determinations.

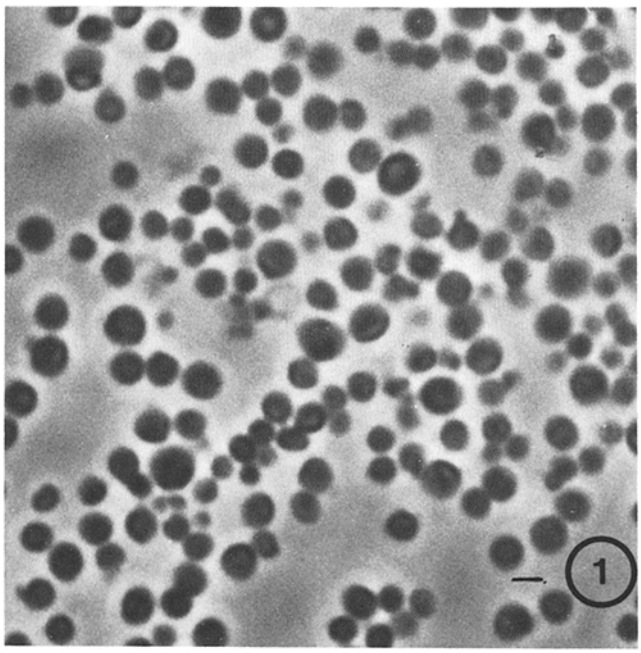

FIGURE 1 Phase contrast micrograph of isolated cortical granules. Granules in buffer B were fixed in 5% TCA, placed between a slide and coverslip, and the preparation was allowed to dry to obtain the granules in a single focal plane. The actual diameter of the granules before fixation and flattening is ~1 μm. Bar, 1 μm. × 4,000.

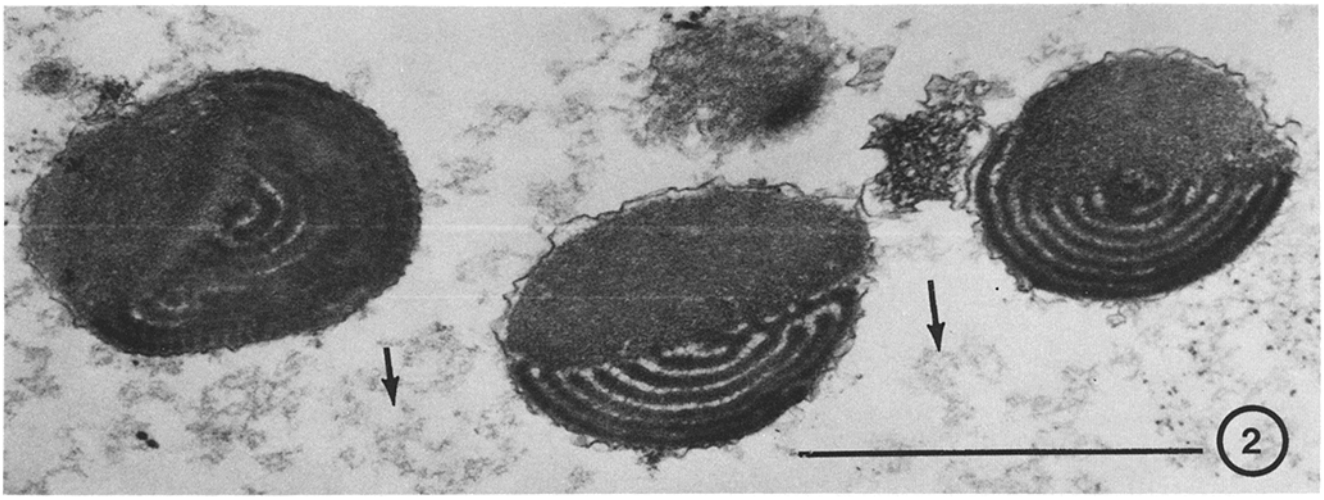

FIGURE 2 Transmission electron micrograph of isolated cortical granules. The CG may have shrunk slightly during dehydration. Note both the lamellar and amorphous areas of the granules. Arrows indicate the precipitate of BSA. Bar, 1 μm. × 54,000.

TABLE II

Enzyme Activities of Egg Homogenates and Isolated Cortical Granules

Enzyme	Specific Activity		Units of activity
	Egg homogenate	Cortical granules	
			min $^{-1}$ · *mg protein* $^{-1}$
5′-Nucleotidase	2.44 ± 0.03	ND‡	nmol P_i released
Adenylate cyclase	32.30 ± 5.85	ND	pmol cAMP formed
Na^+/K^+ ATPase	21.08 ± 4.71	5.03 ± 1.44	nmol P_i released
Acid phosphatase	13.24 ± 1.17	1.35 ± 0.77	nmol P_i released
Glucose-6-phosphate dehydrogenase	62.95 ± 4.22	ND	nmol NADP reduced
Cytochrome c oxidase	0.137 s^{-1}	ND	1st order rate constant (100 μg protein)
Exo-β-(1 → 3)-glucanohydrolase	7.88 ± 2.13	28.79 ± 5.73	nmol glucose released
Ovoperoxidase	61.92 ± 5.74	338.41 ± 3.1	nmol H_2O_2 decomposed
Proteoesterase	0.68 ± 0.09	4.71 ± 0.33	nmol CH_3OH released

* Data represent mean ± SD of at least five different preparations.
‡ Not detectable.

posed of both amorphous and lamellar areas. This species: specific CG morphology is identical to that previously described for *S. purpuratus* (12, 20, 23, 38, 61). Maintenance of the intact CG membrane is observed only when the CG are fixed in BSA. Membranous material associated with the CG is observed in some micrographs; this material could represent CG membrane, plasma membrane, or membrane from other organelles.

Biochemical Criteria of Cortical Granule Purity

MARKER ENZYMES: Whole egg homogenates and CG lysates were compared for the activity of a variety of marker enzymes (Table II). Plasma membrane-associated enzymes such as 5′-nucleotidase (63) and adenylate cyclase (9, 31) are present in the whole egg homogenate but are absent in the isolated CG. The plasma membrane of unfertilized *S. purpuratus* eggs is enriched in Na^+/K^+-ATPase activity (45). This enzyme is present in the isolated CG to 25% of the specific activity of the egg homogenates. Acid phosphatase was used as a lysosomal (22) and egg yolk platelet (24, 66) marker. This enzyme is present in the isolated CG to ~10% of the specific activity of the egg homogenate. This may represent a minor contamination of yolk platelets or may be indicative of the association of this enzyme with the CG. The activity associated with the CG does not represent a *p*-nitrophenylphosphatase activity of the Na^+/K^+-ATPase, because the Na^+/K^+-ATPase, unlike the acid phosphatase, is not active when assayed in the presence of 0.1% Tergitol NPX or in the absence of Mg^{2+}. Furthermore, the Na^+/K^+-ATPase activity is greatly reduced at pH 5, and acid phosphatase activity is not affected by ouabain. Contamination of CG by the cytosolic fraction is negligible, as demonstrated by the absence of glucose-6-phosphate dehydrogenase activity (47, 48). Mitochondrial contamination (cytochrome *c* oxidase) is also negligible.

Glucanase, ovoperoxidase, and proteoesterase activities were selected as enzyme markers because they have been shown to be closely associated with, or localized in, this organelle (8, 23, 26, 44, 45, 66, 67). About 60% of the total glucanase activity is released from the eggs by CG exocytosis at fertilization; the location of the remaining 40% remains unknown (26, 67). These three enzymes are present in higher specific activity (four- to sevenfold) in the CG than in the egg homogenate.

RADIOIODINATION OF INTACT EGGS: To analyze further the purity of isolated CG, the surfaces of intact eggs were radioiodinated and the distribution of label was followed through the CG isolation. As shown in Table III, only about 2–9% of the radioactivity associated with whole eggs and egg lawns is presumed to be associated with protein (Table III *B*, *E*). This conclusion is supported by the low number of TCA-insoluble counts. Since there is essentially no (<0.2%) chloroform:methanol-soluble radioactivity in these fractions (Table

TABLE III

Distribution of Radioactivity in ^{125}I-labeled Eggs

Treatment	Experiment No.	Washed egg suspension*	Egg lawn	Cortical granule lawn	Plasma membrane-vitelline layer	Isolated cortical granules
(A) Total cpm applied to GF/A filters (before extraction)	1	185,321	146,914	4161	4833	414
	2	233,434	220,518	6504	13,102	624
(B) Cpm remaining after TCA, acetone and $CHCl_3$:CH_3OH (% of A presumed to be protein)	1	17,473	12,958	1088	3949	515
		(9.4%)	(8.8%)	(26.1%)	(81.7%)	(100%)
	2	10,259	4658	1681	5066	230
		(4.4%)	(2.1%)	(26.0%)	(38.7%)	(36.9%)
(C) $CHCl_3$:CH_3OH soluble cpm (% of A presumed to be lipid)	1	310	142	32	86	46
		(0.2%)	(0.1%)	(0.8%)	(1.8%)	(11.1%)
	2	472	204	16	184	8
		(0.2%)	(0.1%)	(0.3%)	(1.4%)	(1.3%)
(D) Cpm presumed to be free ^{125}I = A − (B + C) (% free ^{125}I = D/A)	1	167,538	133,814	3041	798	—
		(90.4%)	(91.1%)	(73.1%)	(16.5%)	(0%)
	2	222,703	215,656	4807	7852	386
		(95.4%)	(97.8%)	(73.9%)	(59.9%)	(61.9%)
(E) Cpm remaining after trypsin digestion of filters treated in B (% cpm solubilized by trypsin = 1 − E/B)	1	944	1807	136	211	137
		(94.6%)	(86.1%)	(87.5%)	(94.7%)	(73.4%)
	2	374	127	81	105	39
		(96.4%)	(97.3%)	(95.2%)	(97.9%)	(83.1%)

* Suspensions were washed five times.

TABLE IV

Specific Radioactivity in ^{125}I-Labeled Eggs

Fraction	Experiment No.	cpm/dish*	Specific activity: Not corrected for free ^{125}I‡	Specific activity: Corrected for free ^{125}I§
			cpm/mg protein	
Washed (5 times) egg suspension	1	—	137,723	12,286
	2	—	137,668	5,839
Egg lawn	1	1.57×10^6	136,715	10,382
	2	2.31×10^6	145,235	2,968
Cortical granule lawn	1	11,185	28,795	6,576
	2	19,427	44,328	10,972
Plasma membrane-vitelline layer	1	7,155	161,467	124,927
	2	14,373	243,086	92,098
Isolated cortical granules	1	210	1,989	1,460
	2	325	3,880	1,190

* Value per dish using from 2 to 16 Falcon 3002 culture dishes per fraction.
‡ ^{125}I Associated with unextracted fractions.
§ ^{125}I Insoluble in TCA, acetone, and chloroform:methanol but digested by trypsin (Table III).

III, *C*), it is probable that most (90–98%) of the associated radioactivity is free ^{125}I. The CGL, PM-VL, and CG have considerably greater percentages of ^{125}I-associated protein than the whole egg fractions (Table III *B*, *E*), but they have very little chloroform:methanol-soluble ^{125}I (Table III, *C*). From these data it is possible to calculate the specific activity of the different egg fractions corrected for free ^{125}I (Table IV). The isolation of the PM-VL fraction results in a 8–19 fold increase in specific radioactivity (cpm/mg protein) over the CGL. This agrees well with the 18-fold decrease in protein obtained when collecting the PM-VL from the CGL (Table I). These data suggest that the protein-associated ^{125}I is confined almost exclusively to the PM-VL fraction. This conclusion is supported by the fact that the corrected specific activity of the isolated CG is low (Table IV). If the CG-associated radioactivity in these experiments is due solely to PM-VL, contamination by the PM-VL fraction would be ~1%.

Radiolabeling of Intact and Lysed Cortical Granules

Additional supporting evidence for the intactness of isolated CG is provided by experiments in which intact and hypotonically lysed CG are compared with respect to their efficiencies of radioiodination. Isolated CG are labeled to an average specific activity of 6×10^3 cpm/mg protein and lysed (buffer C) CG to 1.12×10^7 cpm/mg protein. The CG thus appear to be relatively intact with respect to the accessibility of exposed iodinatable groups.

Electrophoretic Characterization of Isolated Cortical Granules

SDS PAGE: The proteins comprising the CGL (Figs. 3 *B* and 4 *B*) are accounted for in the CG (Figs. 3 *C* and 4 *C*) and PM-VL (Figs. 3 *D* and 4 *D*) fractions. A 290,000-dalton PAS-positive protein (Fig. 3 *D*) and a 43,000-dalton protein (Figs. 3 *D* and 4 *D*) appear to be specifically associated with the PM-VL. Most of the proteins associated with the CG and PM-VL fractions are highly enriched as compared with crude egg homogenates (compare lanes *A*, *C*, and *D* of Figs. 3 and 4). The 32,000- and 35,000-dalton bands may be the previously identified major structural proteins of the fertilization envelope (6).

IDENTIFICATION OF COMPONENTS OF THE CORTICAL GRANULE AND PLASMA MEMBRANE-VITELLINE LAYER FRACTIONS: The following results suggest that the 43,000-dalton protein is actin (82, 83). The protein can be extracted from the PM-VL with 0.6 M KI (42, 72), it binds to DNase I-Sepharose and elutes with 3 M guanidine HCl (50, 72), and the eluted material comigrates with purified *Dictyostelium discoideum* actin (71).

In the absence of SDS, the glucanase activity of isolated CG

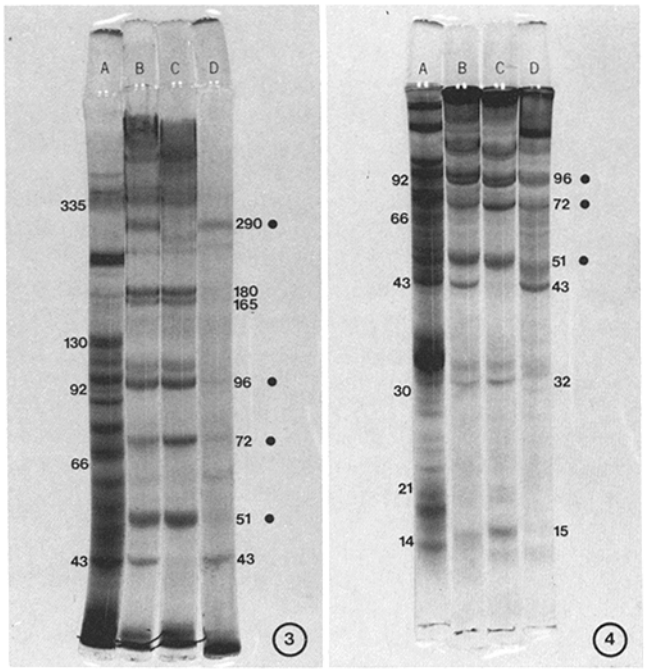

FIGURE 3 SDS-5% PAGE of 100 μg of protein of fractions obtained from the cortical granule isolation procedure. (*A*) whole egg homogenate; (*B*) cortical granule lawn (CGL); (*C*) cortical granules (CG); and (*D*) plasma membrane-vitelline layer (PM-VL). The relative molecular weights of the predominant Coomassie Blue and/or PAS-positive bands (●) are shown on the right. Molecular weight standards were: thyroglobulin (335,000 mol wt); β-galactosidase (130,000 mol wt); phosphorylase a (92,000 mol wt); bovine serum albumin (66,000 mol wt); ovalbumin (43,000 mol wt).

FIGURE 4 SDS-10% PAGE of fractions obtained from the cortical granule isolation procedure. Legend as in Fig. 3. Molecular weight standards were: phosphorylase a (92,000 mol wt); bovine serum albumin (66,000 mol wt); carbonic anhydrase (30,000 mol wt); soybean trypsin inhibitor (21,000 mol wt); and lysozyme (14,300 mol wt).

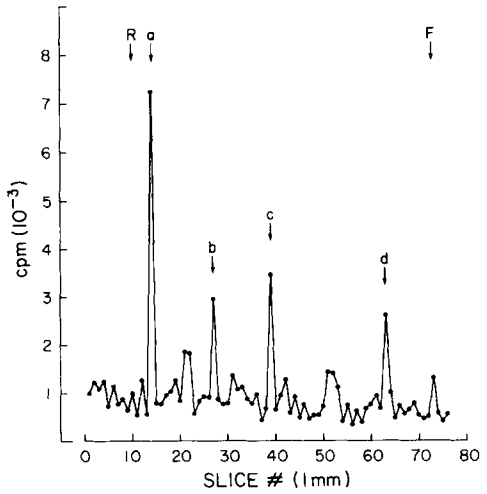

FIGURE 5 Migration of proteoesterase activity obtained from isolated cortical granules on nondenaturing 5% polyacrylamide gels. Samples were prepared and electrophoresed, and gel slices were assayed for enzyme activity. (*R*) beginning of running gel; (*F*) gel front as denoted by the migration of bromphenol blue; (*a–d*) denote positions of the major peaks of proteoesterase activity.

TABLE V

Effects of CG Lysates, Exudates, Trypsin, and SBTI on Bovine Brain Cyclic Nucleotide Phosphodiesterase Activity*

Experiment No.	Incubation condition	Phosphodiesterase activity
		pmol cGMP hydrolyzed/30 min/50 μl enzyme
1	Control	23
	CG lysate	97
	Heated CG lysate (85°C, 30 min)	76
2	Control	50
	CG exudate	
	No treatment	222
	Heated (85°C; 5 min)	152
	+1 mg/ml SBTI	106
	Trypsin	
	4 μg/ml	220
	+1 mg/ml SBTI	38
	40 μg/ml	231
	+1 mg/ml SBTI	40
3	Control	50
	CG lysate	174
	+1 mg/ml SBTI	105

* Soybean trypsin inhibitor.

migrates as one peak on 5% gels with an M_r of 123,000. However, when the sample is incubated with, and run in, 0.1% SDS (without heating), the enzyme migrates as a single peak on both 5% and 10% gels with an M_r of 105,000–110,000. These results agree with published data (77) demonstrating that the purified *S. purpuratus* egg glucanase migrates at M_r 110,000 under similar conditions.

Proteoesterase activity, determined under nondenaturing conditions in 5% gels (Fig. 5), can be detected at a variety of positions. The major peaks of activity may represent aggregate forms of the enzyme (8, 27).

Using CGL preparations Baker and Whitaker (3) demonstrated that trifluoperazine inhibits the Ca^{2+}-mediated discharge of *Echinus esculentus* CG. Since this drug antagonizes many calmodulin-mediated reactions, calmodulin might play a role in CG discharge. Calmodulin is present in sea urchin eggs (7, 25, 33, 55), and antibody to calmodulin blocks the Ca^{2+}-mediated discharge of CGL (73). We found that isolated CG contain a nondialyzable factor that stimulates the activity of calmodulin-deficient bovine brain cyclic nucleotide phosphodiesterase (Table V). Initial determination of the specific activity of this factor demonstrated that it was present in CGL in higher concentrations than in whole egg homogenates. Approximately 22–35% of the activity is heat labile (85°C for 30 min). Since the basal catalytic activity of cyclic nucleotide phosphodiesterases can be increased by limited proteolysis (76, 79), it is possible that part of the CG-associated phosphodiesterase stimulatory activity can be attributed to the CG proteoesterase. Preparations of CG exudate (obtained by activation of eggs with A23187; 81), lysed CG, and crystalline pancreatic trypsin all activate phosphodiesterase activity (Table V). Heat treatment (85°C) of the exudate before incubation with the enzyme partially abolished the stimulating activity. Since the activity of the CG proteoesterase is trypsin-like and is inhibited by SBTI (8, 27, 65), activity that is heat- and SBTI-insensitive is probably not due to the CG protease (Table V). When the unheated CG lysate is incubated with the phospho-

diesterase, only 26% of the activity is inhibited when the free Ca^{2+} in the assay is chelated by EGTA. When the lysate is heated (30 min; 85°C), ~22% of the activity is destroyed and the remaining activity is lost when Ca^{2+} is chelated. These data suggest that there are at least two phosphodiesterase-activating activities with different properties. One activity is heat-sensitive, partially EGTA-sensitive, and is sensitive to SBTI; this factor probably represents the CG proteoesterase. The other activity is heat-stable, EGTA-sensitive and not affected by SBTI; the properties of this activator closely resemble those of calmodulin.

Fig. 6*A* demonstrates that when the unheated CG lysate is applied to a fluphenazine-Sepharose affinity column (13), phosphodiesterase-activating activity elutes in both the flow-through fraction and a fraction that is specifically eluted from the column with EGTA. If the sample is heated before chromatography (30 min; 85°C), the activity in the flow-through fraction disappears but the activity eluted with EGTA remains (Fig. 6*B*). These data suggest that the heat-stable, EGTA-sensitive activity associated with the CG is indeed calmodulin. Once the basic character of the activating factors was determined, the specific activity of calmodulin in the CG and CGL was reexamined. Under conditions where only calmodulin activity is expressed, the specific activity of calmodulin associated with CG, CGL, and PM-VL is not present in concentrations greater than in whole egg homogenates. Identical data were obtained when these egg fractions were prepared using buffers containing either no free Ca^{2+} (EGTA) or 5×10^{-7} M free Ca^{2+}. The calmodulin present in these preparations is probably bound in such a manner so as to be refractory to extraction with EGTA (52). We conclude that calmodulin is not concentrated in the isolated CG, CGL, or PM-VL.

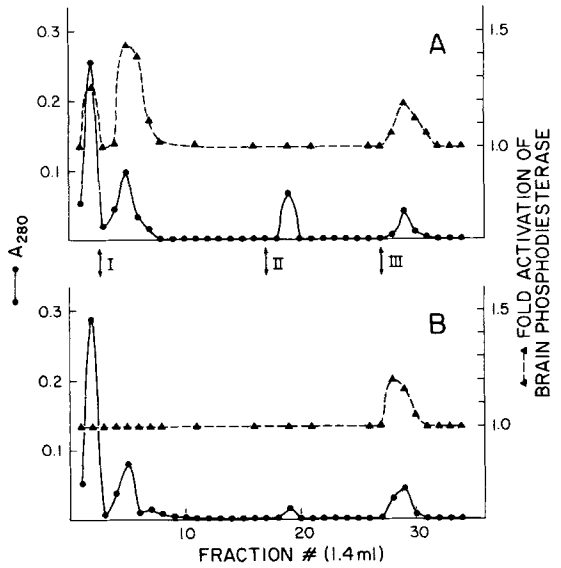

FIGURE 6 Chromatography of the cortical granule lysate on fluphenazine-Sepharose 4B (9 × 0.7 cm) before (*A*) and after (*B*) heating the sample at 85°C for 30 min. The fractions were then assayed for phosphodiesterase stimulating activity using the calmodulin-deficient brain enzyme. Before chromatography, samples were dialyzed (4°C) extensively against the column equilibration buffer (10 mM HEPES, 0.5 mM $CaCl_2$, pH 7.0). The samples were applied and the column was then washed with: (*I*) column equilibration buffer; (*II*) column equilibration buffer containing 0.5 M NaCl; and (*III*) 10 mM Tris, 5 mM EGTA, pH 8.0.

DISCUSSION

Our purpose was to develop a method to isolate intact CG free in suspension, devoid of other cellular constituents, and in large enough quantities to permit biochemical characterization. The isolated CG are a relatively pure population as judged by light and electron microscopy and by enzymatic analysis. The presence of Na^+/K^+ ATPase activity in the isolated CG was initially of concern because Kinsey et al. (45) had demonstrated the presence of this enzyme in the egg plasma membrane. This, along with the fact that in some instances there is membranous material associated with the CG, suggested that there might be plasma membrane contamination in the CG preparation. Although this cannot be unequivocally excluded, we have observed that contamination by the ^{125}I-labeled PM-VL fraction is minimal (Table IV) and that contamination by plasma membrane marker enzymes (5′-nucleotidase; adenylate cyclase) is negligible. SDS PAGE of the different egg fractions demonstrates that the CG and PM-VL have distinct electrophoretic patterns and that protein carry-over into the CG from the PM-VL is not evident by Coomassie staining. This level of plasma membrane contamination is comparable to that observed by Decker and Kinsey (21) using a different CG isolation procedure. It is possible that ATPase activity may be specifically associated with the CG. There is substantial evidence to indicate that ATPases function in the sequestration of intragranular components of secretory granules (1, 40, 41) and also mediate secretory granule exocytosis (19, 56–59). Contamination of the CG by mitochondrial and cytoplasmic components is undetectable.

As expected, the isolated CG contain the three enzymes known to be associated with sea urchin egg CG in specific activities higher than in the whole egg homogenate. The enrichment in specific activity (four- to sevenfold) is not as great as the 10- to 11-fold enrichment reported by Schuel et al. (66) using the glucanase as a CG marker. Such preparations of CG will be useful for studying the properties of these enzymes before their release into sea water during egg activation.

The isolated CG appear to be morphologically intact. Additional evidence for this integrity comes from the data demonstrating that intact CG are not radioiodinated to a very high specific activity, although the capacity for radiolabeling is high. The present isolation procedure results in a higher percentage of morphologically intact CG than has been obtained by previously published procedures (65, 66, 68), and yields CG that are similar in morphology to those recently described by Hylander and Summers (38). The inability of intact CG to be radiolabeled to high specific activity poses an interesting question as to the structure of the exposed region of these secretory granules. However, we have not attempted to iodinate the CG by other procedures, so it is possible that other labeling methods might yield different results.

Although the morphological intactness and iodination studies lead to the presumption that the granules are well preserved, isolated CG in buffer B lose their property of immediate disruption (lysis) when exposed to micromolar concentrations of Ca^{2+} and thus have lost their physiological intactness. However, they will disrupt, with concomitant release of their contents (glucanase, proteoesterase), when exposed to millimolar free Ca^{2+} (46). The reasons for this loss of Ca^{2+} sensitivity are not known. The loss of a specific factor required for granule plasma membrane interaction and fusion (16–18, 73) or the physical disruption of CG membrane-plasma membrane interactions might contribute to this loss of Ca^{2+} sensitivity.

During the development of this isolation procedure, we observed that certain conditions had to be met for the successful isolation of stable CG. Granule stability was enhanced by the inclusion of both 5×10^{-7} M free Ca^{2+} and high concentrations of sodium isethionate. It is not known whether there are Ca^{2+} binding sites on the CG membrane, but it is possible that such sites must be filled to maintain membrane integrity. Since sodium isethionate competes with Cl^- for chromaffin granule membrane-associated anion transport sites (57) and inhibits the Cl^--mediated release of catecholamines from these granules (35, 57), it is possible that this anion is acting through a similar mechanism in the CG.

Characterization of the CG and PM-VL shows that actin is associated only with the PM-VL fraction, which is consistent with other reports (5, 72, 83). However, it is important to note that the specific localization of actin to a particular egg fraction is always subject to interpretation because of the dramatic effects that buffers of various ionic composition have on the solubility of this protein. The glucanase and proteoesterase activities have also been localized in gels of the CG lysate, and the molecular weights of these enzymes agree well with previous reports (27, 77). Further characterization of and differentiation between the CG membrane and intragranular components using these isolation methods might contribute to our understanding of the mechanisms involved in the exocytotic release of CG contents. Preparation of CG membrane might also be accomplished using this isolation procedure.

Considerable attention has been given to the determination of calmodulin associated with the CGL. Recent reports suggest that it may play a role in CG exocytosis (3, 73). Since there is evidence in other cell types for the specific localization of calmodulin in isolated purified subcellular fractions (30, 52), we investigated the localization of calmodulin in the CGL, CG, and PM-VL. Initial experiments suggested that such a specific localization was present, but this was subsequently disproven when it was demonstrated that the proteoesterase activity of the CG could partially mimic the effects of calmodulin to activate the cyclic nucleotide phosphodiesterase. The data obtained do not preclude a role for calmodulin in CG exocytosis. However, experiments demonstrating a direct effect of calmodulin on CG exocytosis are required to confirm such a positive association.

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