In Vitro Reconstitution of Exocytosis from Plasma Membrane and Isolated Secretory Vesicles

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ABSTRACT We describe the reconstitution of exocytotic function through recombination of purified cortical secretory vesicles (CVs) and plasma membrane from sea urchin eggs. CVs were dislodged from a cell surface complex preparation by gentle homogenization in an isotonic dissociation buffer, and purified by differential centrifugation. CV-free plasma membrane fragments were obtained by mechanically dislodging CVs from cortical lawn (CL) preparations with a jet of CL isolation buffer. This procedure produced a "plasma membrane lawn" preparation, consisting of plasma membrane fragments attached via their vitelline layer (an extracellular glycocalyx) to a polylysine-coated microscope slide. When freshly prepared CVs were incubated with plasma membrane lawns, CVs reassociated with the cytoplasmic face of the plasma membrane, forming an exocytotically competent, reconstituted cortical lawn (RL). Exocytosis in RLs was monitored by phase-contrast microscopy, and quantitated with a sensitive microphotometric assay. Half-maximal exocytosis in RLs occurred at 18.5 μM free Ca^{2+}; half-maximal exocytosis in control lawns occurred at 5.7 μM free Ca^{2+}. Greater than 90% of the purified CVs that were not attached to a plasma membrane lawn remained intact when bathed in a buffer containing millimolar Ca^{2+}. This result excluded the possibility that Ca^{2+}-triggered CV lysis was responsible for our observations, and confirmed that the association of CVs with the plasma membrane was required for exocytosis in RLs. Evidence that the Ca^{2+}-stimulated release of CV contents in CLs and RLs is the in vitro equivalent of exocytosis was obtained with an immunofluorescence-based vectorial transport assay, using an antiserum directed against a CV content protein: stimulation of RLs or partially CV-depleted CLs with Ca^{2+} resulted in fusion of the CV and plasma membranes, and the vectorial transport of CV contents from the cytoplasmic to the extracytoplasmic face of the egg plasma membrane.

Efforts to elucidate the molecular mechanism of exocytosis have been hampered by the failure to reconstitute this process in vitro, through recombination of plasma membrane (PM)[1] and secretory vesicle fractions (see references 1 and 2 for an appraisal of attempts to reconstitute exocytosis in vitro). A partial resolution of this problem was provided by the discovery that cortical fragments of the eggs of various species of sea urchin comprise an exocytotically competent complex consisting of the vitelline layer, the plasma membrane, and its attached cortical secretory vesicles (CVs).

Procedures have been developed for the preparation of cortical fragments both in suspension (cell surface complex [CSC] preparation [3]) and attached to polycation-coated supports (cortical lawn [CL] preparation [4]). The morphology of these preparations has been shown to change dramatically in response to micromolar-free Ca^{2+}. The most dramatic change is the disappearance of the CVs, concomitant with the formation of a fertilization envelope-like structure (5, 6). Direct evidence for fusion of CVs with the PM has been obtained by electron microscopic analysis of (*a*) fixed and embedded samples (7), and (*b*) critical point–dried and rotary-shadowed samples (8). As expected, this Ca^{2+}-triggered morphologic change is accompanied by the release of CV content enzymes: ovoperoxidase (9) and β-1,3 glucanase (10).

Although the CSC and CL preparations are exocytotically competent, their usefulness in investigations of the molecular basis of exocytosis is limited because the CVs and the PM cannot be independently manipulated. A reconstituted exo-

[1] *Abbreviations in this paper:* CL, cortical lawn; CSC, cell surface complex; CV, cortical vesicle; NEM, N-ethylmaleimide; PM, plasma membrane; RL, reconstituted lawn; SW, sea water; TAME, *N*-tosyl-L-arginine methyl ester.

cytotic system in which purified CV and PM fractions are recombined to form an exocytotically competent complex would surmount this difficulty, and pave the way for investigations at the molecular level. In this report we describe the development and initial charcterization of such a system.

MATERIALS AND METHODS

Materials: *Strongylocentrotus purpuratus* was purchased from Marinus, Inc. (Ingelwood, CA) and maintained at 12–15°C in a refrigerated aquarium containing Instant Ocean seawater (SW) from Aquarium Systems (Mentor, OH). Bovine serum albumin (BSA, fraction V), EGTA, fluorescein isothiocyanate–conjugated goat anti-rabbit IgG, HEPES, *N*-ethylmaleimide (NEM), PIPES, and poly-L-lysine (molecular weight, 2–3 × 10^5) were purchased from Sigma Chemical Co. (St. Louis, MO). Agarose (Seakem, ME) was obtained from FMC Corporation, Rockland, ME. Coomassie Brilliant Blue G-250 was from Eastman Kodak Co., Rochester, NY. [^{32}P]ATP and [^{3}H]-*N*-tosyl-L-arginine methyl ester ([^{3}H]TAME) were purchased from Amersham Corp., Arlington Heights, IL. Rabbit antiserum to sea urchin hyalin was a generous gift from Dr. D. R. McClay, Duke University, Durham, NC. All other reagents were of the highest grade available.

Preparation of CLs and PM Lawns: Eggs were shed into 0.45 μm filtered SW by intracoelomic injection of 0.5 M KCl, filtered through a 125-μm mesh nylon screen (Small Parts, Inc., Miami, FL), and washed by three cycles of centrifugation and resuspension in fresh SW. A 20% suspension (vol/vol) of eggs in SW was maintained on ice and dejellied, immediately before use, by two washes with 502 mM NaCl, 10 mM KCl, 2.5 mM $NaHCO_3$, 25 mM EGTA pH 8.0.

CLs were prepared at room temperature on polylysine-coated glass, as follows. Sample chambers were constructed from 25 × 75 × 1-mm glass slides, by gluing two narrow strips of #1 glass coverslip (Corning Glass Works, Corning, NY) to the edges of the slide with epoxy glue. Polylysine (1 mg/ml) was applied to the center of the slide chamber, incubated for 3 min, then rinsed with a stream of cold PKME buffer (50 mM PIPES, 425 mM KCl, 10 mM $MgCl_2$, 10 mM EGTA, pH 6.8) delivered from a hand-held wash bottle. A slurry of dejellied eggs (20% vol/vol, in SW) was applied to the treated slide and allowed to settle and attach for 3 min. The slide with attached egg monolayer was gently rinsed, then sheared with PKME buffer from the wash bottle, leaving cortical fragments of the eggs attached via their extracytoplasmic face.

Preparation of PM lawns was achieved by subjecting the CL to further shearing with PKME buffer. A stream of buffer was directed across the CL to dislodge CVs from a region of the CL, or from the entire CL, as indicated. Removal of CVs was accompanied by visible clearing of the translucent CL. This phenomenon provided a convenient method for gauging the extent of CV removal during the shearing procedure. A 22-mm^2 coverslip was placed on the coverslip shims forming a 30–50-μl perfusion chamber. The extent of CV removal was routinely confirmed by phase-contrast microscopy. All subsequent washings, buffer exchanges, and additions were achieved by carefully placing a 2-cm^2 filter paper wick (Whatman Inc., Clifton, NJ) on one end of the sample chamber and evenly perfusing 100 μl by capillary action from the opposite side of the chamber. In some experiments, CLs and RLs were prepared on 22-mm^2 coverslips to facilitate preparation and handling.

CSC was prepared in suspension by a modification of the method of Detering et al (3), as described by Jackson et al. (11). CSC lawns were prepared by allowing 50 μl of suspended CSC to attach to coverslips coated with polylysine as in CL preparation, and gently shearing with a jet of PKME buffer to remove unattached portions of CSC and produce flat lawns. Free Ca^{2+} concentrations were calculated as previously described (11).

CV Preparation: CVs were prepared from CSC, as follows: CSC in PKME buffer (A_{400} = 20) was pelleted by centrifugation for 3 min at 250 *g*. The CSC pellet was resuspended to A_{400} = 10 in ice cold KEA buffer (450 mM KCl, 5 mM EGTA, 50 mM NH_4Cl, pH 9.1), and 3-ml aliquots were manually homogenized with five strokes in a Potter-Elvejhem tissue grinder (4-ml capacity). This resulted in release of large numbers of CVs from the CSC, without severely fragmenting the remaining CV-depleted CSC fragments. The suspension was centrifuged twice at 250 *g* for 3 min in a refrigerated centrifuge to separate large CSC fragments from dislodged CVs. The CV-rich supernatant fraction contained no gross contamination with CSC fragments, and CVs in the fraction were judged to be substantially monomeric by phase-contrast microscopy. CVs were stored on ice and used within 1 h for RL preparation. Prolonged storage in KEA buffer resulted in significant CV lysis.

Enzyme Assays: CV proteoesterase activity was assayed as described by Detering et al. (3) using [^{3}H]TAME as a substrate. ATPase activity was assayed at 25°C for 30 min. Samples (250 μl) were buffered with 50 mM HEPES, pH 7.2, and contained 450 mM NaCl, 20 mM KCl, 2.5 mM EGTA, 5.0 mM $MgCl_2$, and 1.0 mM ATP containing 1.0 μCi [^{32}P]ATP. The reaction was quenched with 1 vol of ice cold 10% trichloroacetic acid. The precipitated protein was pelleted by centrifugation, and aliquots of the supernatant were assayed for $^{32}P_i$. The procedure described by Marsh (12) was used to separate $^{32}P_i$ from unhydrolyzed ATP. Aliquots of the $^{32}P_i$-containing butanol phase were counted in Aquasol (New England Nuclear, Boston, MA). Both the ATPase assay and the TAME hydrolase assay were shown to be linear with time and enzyme concentration. Protein was determined using the Coomassie Brilliant Blue G-250 method with BSA as a standard (13).

Preparation of RLs: A freshly prepared suspension of CVs was neutralized to pH 6.8 by the addition of enough 1 M PIPES, pH 6.1, to bring the final solution to 40 mM PIPES. 100 μl of this suspension was immediately applied to a freshly prepared PM lawn, either by flushing into the perfusion chamber containing the slide-bound PM lawn, or by pipetting the suspension onto a PM lawn–containing coverslip. After 10 min at room temperature, the unbound CVs were removed from the perfusion chamber by five washes (100 μl each) with PKME buffer. RLs prepared on coverslips were washed by simply dipping the coverslip repeatedly into sequential beakers of PKME buffer. Care was taken at all stages of the CL, PM lawn, and RL preparation to prevent drying.

Integrity of Isolated CVs Upon Treatment with Millimolar Concentrations of Ca^{2+}: Immobilization of the isolated CVs allowed assessment of their morphological integrity in response to applied calcium. This was achieved in two ways. First, CVs were prepared in KE_2A (450 mM KCl, 2 mM EGTA, 50 mM NH_4Cl, pH 9.1), and neutralized to pH 6.8 by the addition of 40 mM PIPES as above. 100 μl of neutralized CV suspension was pipetted into a single well slide whose well had been precoated with 100 μl of 1% agarose in the same buffer (referred to as PKE_2A). The lower EGTA concentration (2 mM) used in this experiment allowed the 40 mM PIPES to adequately buffer the pH during the addition of the calcium solution. The well was covered with a coverslip, and centrifuged at 250 *g* for 3 min. This resulted in deposition of numerous CVs onto the agarose surface of the well. The well was filled with PKE_2A and a coverslip remounted. Phase-contrast micrographs were taken of an individual field of agarose-bound CVs before, during, and 5 min after the infusion of sufficient 16 mM $CaCl_2$ (in PKE_2A, pH 6.8) to bring the final Ca^{2+} concentration to 2 mM. CVs were counted on micrographs and scored for degree of aggregation (monomer, dimer, or oligomer [3 or more]), and morphology (lysed or intact). The morphology of monomeric CVs was easily scored due to the loss of phase refractility upon lysis. Dimers and oligomers underwent a gross morphological change upon "lysis" that transformed them into nonrefractile amorphous masses containing the phase-dense CV content material of the aggregated CVs.

The second way of assessing the morphological integrity of the CVs in response to applied calcium was based on our observation that while isolated CVs at pH 6.8 exhibited little binding to slides or coverslips coated with polylysine, they bound strongly to the surface of clean, uncoated glass. Thus, 100 μl of a neutralized suspension of CVs was applied to a glass slide perfusion chamber (which had been cleaned with methanolic KOH and exhaustively rinsed) and allowed to settle and bind for 10 min at room temperature. Unbound CVs were washed out of the chamber by perfusion with PKME buffer as described for RLs. The slide-bound CVs were then challenged by the addition of PKME buffer containing 2 mM free Ca^{2+}. Phase-contrast micrography and scoring of the CVs were performed as above. The results obtained with this method were virtually identical to those obtained using the agarose technique.

Immunofluorescence Assay for Vectorial Transport of CV Contents Across the Plasma Membrane: This assay used rabbit antiserum prepared against purified hyalin (from *Arbacia punctulata*) and cross-reactive with *S. purpuratus* (14). The serum was absorbed with CSC to remove low levels of reactivity to the cytoplasmic surface of PM lawn, CL, and RL preparations. Absorptions were performed as follows: a pellet of CSC containing ~0.5 mg protein was resuspended in 1.0 ml of diluted serum (1:5 in PKME buffer, pH 6.8). After a 30-min incubation on ice, CSC was removed by low speed centrifugation (250 *g* for 3 min). The absorbed serum was collected, centrifuged in a microfuge at 15,000 *g* for 3 min and filtered through a 0.22-μm Millex GV syringe filter (Millipore Corp., Bedford, MA).

"Sparse CLs," with only a few bound CVs, were prepared by shearing CLs with a jet of PKME buffer until most but not all CVs had been dislodged. A coverslip containing the RL or sparse CL to be examined was mounted on a perfusion chamber and microscopically observed during the addition of a PKME buffer containing 44 μM free Ca^{2+}. Upon initiation of CV release, the chambers were immediately flushed with 1% glutaraldehyde (EM grade, Ladd Research Industries, Inc., Burlington, VT) in PKME buffer, pH 6.8. After 10

min of fixation at room temperature, the coverslips were removed from the chambers, placed in alumina coverglass racks (Thomas Scientific, Philadelphia, PA) and immersed in $PK_{475}ME$, pH 6.8 (same as PKME, but with 475 mM KCl to prevent osmotic imbalance during the glutaraldehyde removal). After 1 h in this solution, the racks were transferred to PKME and held at 4°C for 2 h or overnight. Where indicated, membranes were disrupted by immersing coverslips containing the fixed RL or sparse CL samples in PKME, pH 6.8, containing 0.5% Triton X-100 (Mallinckrodt Inc., St. Louis, MO) for 15 min, followed by two washes (10 min each) in PKME buffer.

Immunofluorescence was carried out as follows: the coverslips were placed in a humid chamber, and 100 μl of absorbed anti-hyalin antiserum (final dilution 1:20 or 1:40 in PKME containing 5% normal goat serum), or a control serum at the same dilution, was added to each coverslip. After 1 h, the coverslips were placed in a coverglass rack and washed twice in PKME buffer. The washed coverslips were returned to the humid chamber and incubated 30 min with 100 μl of fluorescein isothiocyanate—conjugated goat anti-rabbit IgG (diluted 1:500 in PKME buffer containing 5% goat serum). After two additional washes in PKME buffer, the coverslips were mounted for microscopy. Paired phase-contrast and fluorescence micrographs were taken of individual RL or sparse CL fragments.

Microphotometric Assay: This assay is similar to that described by Zimmerberg (14a). Briefly, when viewed under low power darkfield optics, the lawns scatter light due to the presence of the individual CVs. The amount of scattered light is measured by a sensitive photodiode/transistor assembly mounted on the camera tube of the microscope, and displayed on a potentiometric chart recorder. A decrease in the output voltage of the photodiode occurs upon addition of calcium-containing buffers. We have shown that this microphotometric response is linear with respect to the extent of the cortical reaction (15).

Photography: Phase-contrast and immunofluorescence micrographs were taken on Panatomic X or Tri-X film (Eastman Kodak Co.), respectively, using a Zeiss universal microscope and 20× plan achromat (Nikon) or 40× achromat water immersion (Zeiss) phase-contrast objectives and using phase-contrast, darkfield, or epifluorescence optics, as indicated. Measurements were made with a stage micrometer.

RESULTS

Purification of CVs

Our CV purification procedure is based on the observation of Hylander and Summers (16) that the CVs in an intact egg can be dissociated from the PM by a two-step procedure in which the eggs are first incubated in buffers containing ammonium chloride, procaine, or tetracaine at pH 9.0, and then centrifuged to stratify the cellular contents. This observation suggested that CVs could be gently dislodged from CSC, without seriously reducing the size of the CV-depleted CSC fragments, thereby facilitating the rapid separation of released CVs from the much larger CSC fragments.

The procedure we have devised consists of the gentle homogenization of CSC in an isotonic, ammonia-containing buffer at pH 9.1, followed by two cycles of differential centrifugation. The supernatant fraction from the second centrifugation step is our CV suspension. CVs prepared by this procedure were determined to be morphologically intact and primarily monomeric with only a few aggregates (Fig. 1).

The extent of PM contamination of CV preparations was ascertained by assaying ouabain-inhibitable (Na^+, K^+)ATPase activity (12). As reported in Table I, the (Na^+, K^+)ATPase activity of our CV preparations was 10% ($n = 6$) of the CSC fraction. This decrease in (Na^+, K^+)ATPase activity was not an artefact of the pH 9.1-NH_4Cl extraction procedure: CSC controls that had been subjected to the pH 9.1-NH_4Cl incubation procedure lost no activity.

As a marker for CV contents, we assayed the TAME hydrolase activity of the CV proteoesterase (Table I). This enzyme, whose function is to modify the surface of the fertilized egg (17), was found to be slightly enriched (119%) in purified CV fractions. The small extent of CV proteoesterase enrichment of CV fractions relative to CSC was expected since CVs comprise the bulk of CSC protein (18). Based on the recovery of TAME hydrolase activity, we calculate a yield of 35.3 ± 11.5% (0.28 ± 0.06 mg CV protein/mg CSC protein, $n = 6$).

Vis-a-vis previously reported procedures (18, 19), our procedure is rapid and can be easily scaled up. Most importantly, CVs freshly prepared by this procedure are capable of reassociating with PM lawns to form exocytotically functional CV–PM complexes.

Reconstitution of Exocytotically Functional CV–PM Complexes

As a source of PM for reconstitution experiments, we devised a procedure for producing PM lawns. These preparations consist of fragments of egg plasma membrane attached via their extracytoplasmic surface to a polylysine coated slide (Fig. 2*A*).

PM lawns were prepared by dislodging CVs from a CL with a jet of isotonic buffer directed across the CL surface. When observed by phase-contrast microscopy, the CV-free PM fragments that comprise the PM lawn are clearly seen (Fig. 2*A*).

For purposes of comparison, we removed CVs from most but not all of the PM fragments in the experiment shown in Fig. 2. This permitted us to locate a microscopic field containing a PM fragment that had been completely stripped of its CVs, as well as a control fragment that retained many endogenous CVs. (Actually, a few CVs remain on the denuded fragment. This is intended to show that the PM was not stripped off by the shearing procedure, leaving the vitelline remnant.)

A reconstituted CV–PM complex, or reconstituted lawn (RL), was prepared by incubating a freshly prepared CV suspension, at pH 6.8, with a PM lawn. After a 10-min incubation at room temperature, unattached CVs were removed by several wash cycles. The resultant RL (Fig. 2*B*) was comprised of PM fragments decorated with monomers, dimers, and small aggregates of CVs. A few CVs were observed to bind directly to the polylysine-coated slide surface between PM fragments; however, the density of CVs bound to PM fragments greatly exceeded the density of CVs bound directly to the slide. Close inspection of Fig. 2, *A* and *B*, reveals that some additional CVs also reassociated with the partially stripped control fragment in the lower right-hand corner of these panels.

Fig. 2, *C* and *D*, illustrates the state of the reconstituted cortical fragment shown in *B*, 5 and 30 s after 2 mM free Ca^{2+} was washed into the slide chamber. The most striking observation is that within 5 s (*C*) the CVs released their contents and lost their refractility. Dispersal of the CV contents occurred after release, and continued through the 30-s time point (*D*). Over a period of several minutes, the contents of released CVs appeared to merge together. The control cortical fragment, in the lower right-hand corner of *A–D*, was observed to undergo morphological changes qualitatively identical to the reconstituted fragment. Note that CVs associated with the PM released their contents, while CVs bound directly to the polylysine-coated slide remained intact.

The morphology of an RL that was triggered with 44 μM free Ca^{2+} is shown in Fig. 3. The extent of reaction and the

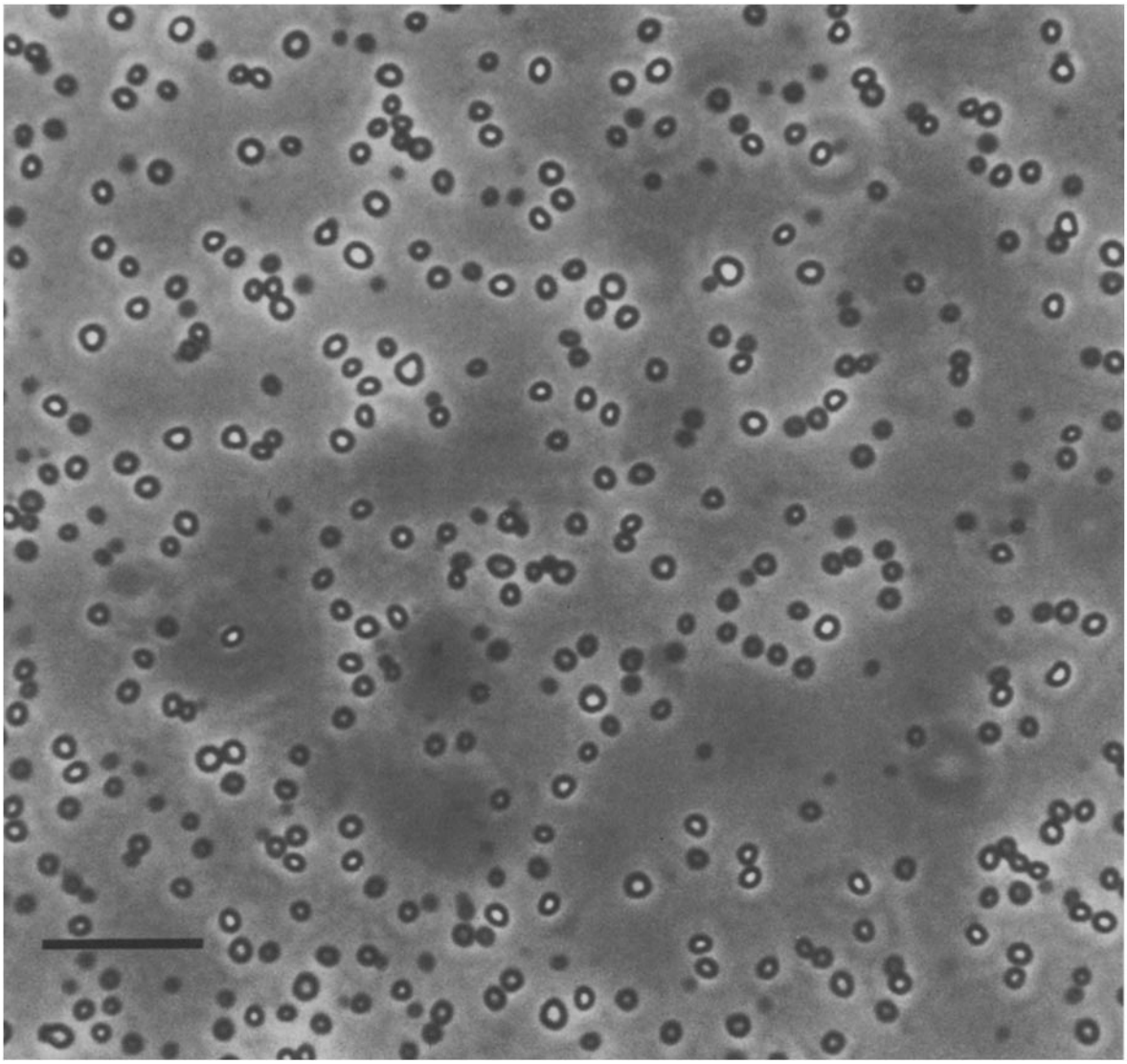

FIGURE 1 Purified cortical vesicles. CVs were isolated from CSC as described in Materials and Methods, applied to a perfusion chamber in KEA buffer, pH 9.1, and allowed to settle for 10 min. (Phase-contrast.) Bar, 10 μm.

TABLE I. *Enzyme Activities of the Cell Surface Complex and Purified Cortical Vesicles*

	(Na^+, K^+)ATPase		Proteoesterase	
Cellular fraction	Specific activity*	Relative activity	Specific activity‡	Relative activity
Cell surface complex	176 ± 65 (6)	1.00	3019 ± 566 (6)	1.00
Cortical vesicles	17.6 ± 15.0 (6)	0.10	3595 ± 662 (6)	1.19

* The specific activity of ouabain-sensitive (Na^+, K^+)ATPase is expressed as nmoles of [^{32}P]ATP hydrolyzed per mg protein per h ± average deviation of the mean (number of independent determinations).

‡ Proteoesterase activity is expressed as nanomoles of ^{3}[H]TAME hydrolyzed per mg protein per h ± average deviation of the mean (number of independent determinations). Individual preparations were assayed in quadruplicate, for ATPase activity, and in duplicate for proteoesterase activity.

overall morphology was the same at 2 mM and 44 μM Ca^{2+}. In both cases, the fusion of monomeric CVs with the PM is clearly visible.

There are three potential mechanistic explanations for the Ca^{2+}-triggered release of CV contents observed in Figs. 2 and 3. (*A*) Release may result from Ca^{2+}-triggered lysis of the CVs and may bear no relationship to the reassociation of CVs with the PM. (*B*) Release may result from Ca^{2+}-triggered fusion of the CVs and PM, and may therefore require the reassociation of CVs with the PM. (*C*) Release may result from a two-step process of CV–CV fusion, followed by lysis. This third explanation can be dismissed, at least in part, on the basis that the monomeric CVs attached to PM lawns released their contents without first fusing with other CVs (compare *B* and *C* in Figs. 2 and 3).

To determine which of these explanations was correct we quantitated the response of monomeric, dimeric, and oligomeric CVs to millimolar concentrations of Ca^{2+} in the absence of PM. CVs that had been rendered stationary by gentle centrifugation onto a bed of agarose were photographed before and after the free Ca^{2+} concentration was raised to 2.0 mM, as described in Materials and Methods. Analysis of these

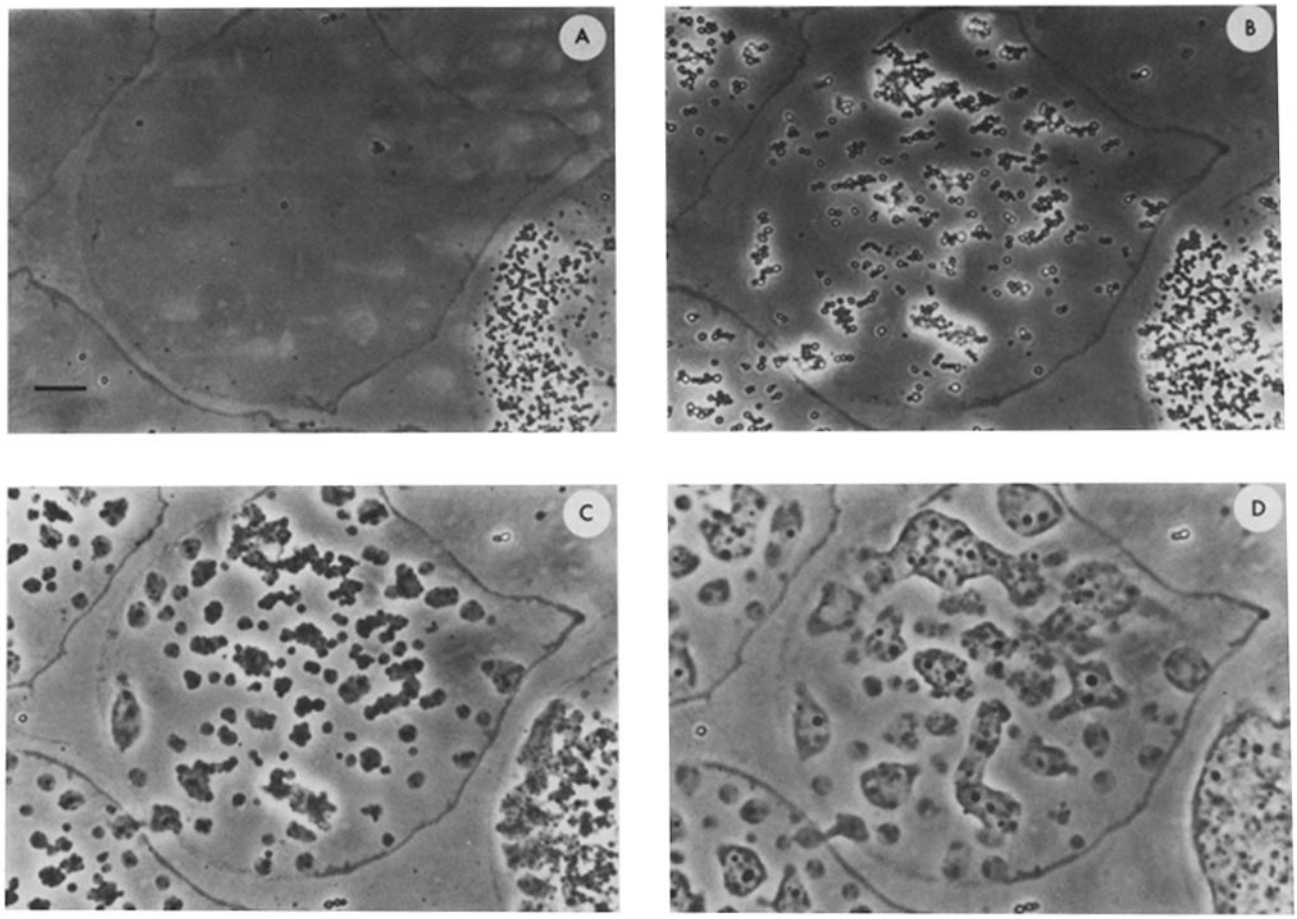

FIGURE 2 Reconstituted cortical lawns and their response to 2 mM Ca^{2+}. A PM lawn was prepared by dislodging CVs from a CL as described in Materials and Methods. A field containing both denuded and intact cortical fragments (for comparative purposes) was located and photographed (*A*). Subsequent steps were performed with the sample in place on the microscope stage so that the same field could be followed throughout the experiment. (*B*) RL formed when CVs were applied at pH 6.8, allowed to bind for 10 min, and washed to remove unbound CVs. (*C* and *D*) Same RL, 5 and 30 s, respectively, after treatment with buffer containing 2 mM free Ca^{2+}. Bar, 10 μm.

photographs allowed us to observe an individual CV, or CV aggregate, and confidently score its reaction to Ca^{2+}. If the first explanation were correct, and Ca^{2+}-triggered lysis was responsible for the release reaction, then all of the CVs should lyse when bathed in Ca^{2+}. If the second explanation were correct, the CVs should remain intact.

The accumulated results of several experiments of this type are presented in Fig. 4. 90.2% of the monomeric CVs remained intact after 5 min in 2.0 mM free Ca^{2+}, while 6% were lysed. A small fraction of the monomers (3.8%) were missing from micrographs taken after Ca^{2+} addition, presumably because they had been dislodged.

The stability of the monomeric CVs in this experiment clearly demonstrated that Ca^{2+}-triggered CV lysis cannot account for the release reaction in RLs. Furthermore, since the monomeric CVs of an RL release their contents without first fusing with another CV (Figs. 2 and 3), the third explanation (CV–CV fusion followed by lysis) cannot account for the release of CV contents from monomers. We conclude that monomeric CVs must be associated with the PM in order to undergo the Ca^{2+}-triggered release reaction.

For dimers (8.7% lysis) and aggregates (30.5% lysis), the interpretation of the data in Fig. 4 is more complicated. The probability that a CV aggregate will lyse when bathed in millimolar concentrations of Ca^{2+} increased with the size of the aggregate. This may mean that the probability of CV–CV fusion increases with the number of CV–CV contacts. Alternatively, it is possible that large CV aggregates form around fragments of PM that contaminate the CV preparation; hence, fusion of large CV aggregates may be PM induced. In any case, the fact that most dimers and oligomers do not lyse when bathed in millimolar concentrations of Ca^{2+} suggests that most of the dimeric and oligomeric CV complexes of an RL may also require attachment to the PM for optimal fusion.

As a control for these experiments, we showed that a CL preparation that had been completely embedded in agarose (by dipping it into a beaker containing 1% agarose, T = 40°C) remained active when challenged with Ca^{2+}. In addition, we obtained nearly identical results when we quantitated the Ca^{2+}-triggered lysis of CVs bound to uncoated glass coverslips.

We also investigated the possibility that the results presented in Figs. 2 and 3 might be an artefact of the buffer conditions used in the CV preparation. This was accomplished by purifying CVs (as described) in a neutral buffer (PKME, pH 6.8) and using these for the preparation of RLs. CVs prepared by this procedure exhibited a significantly greater degree of aggregation than CVs prepared by the pH 9.1 procedure; consequently the RLs contained a greater

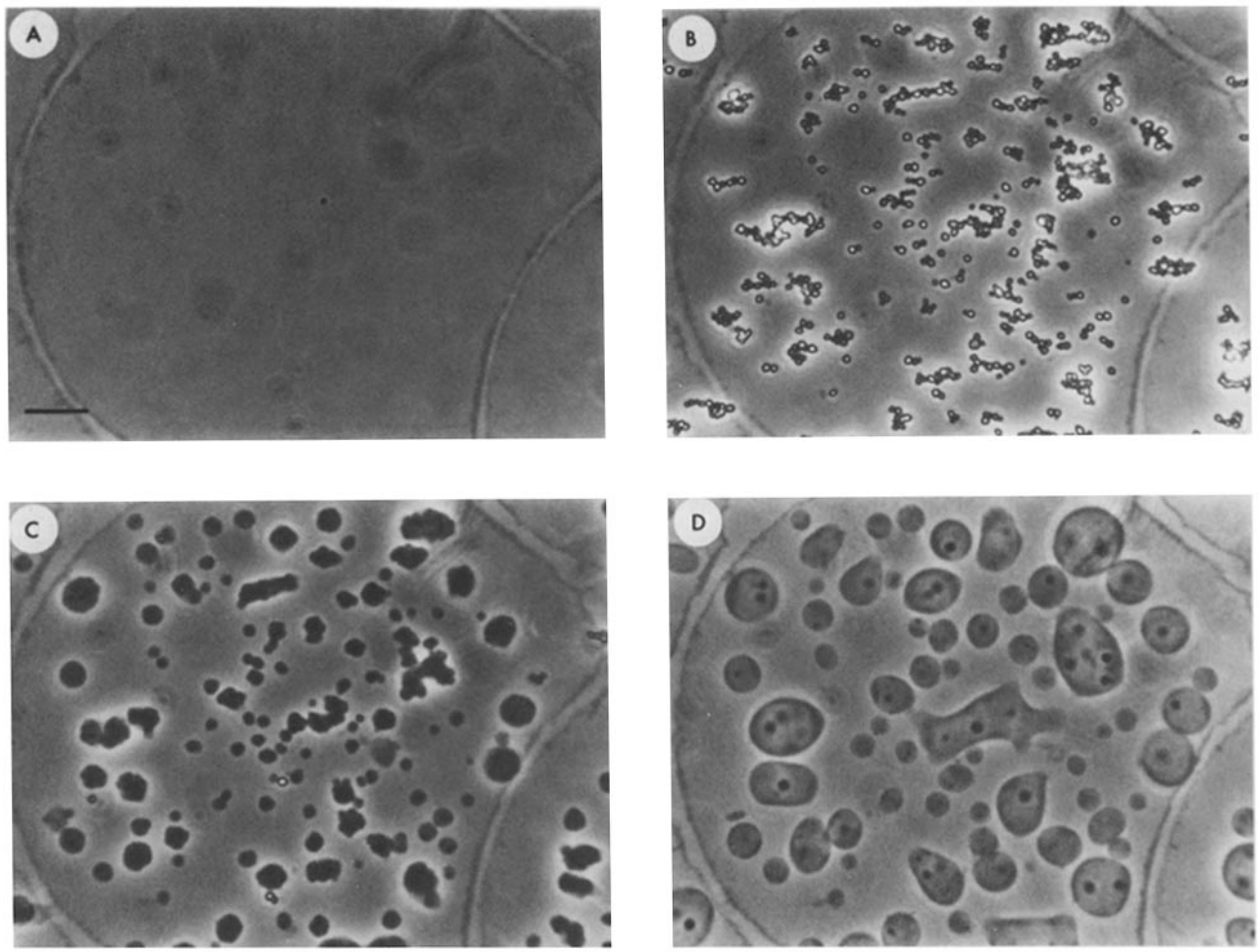

FIGURE 3 The response of RLs to 44 μM Ca^{2+}. A PM lawn was prepared by dislodging CVs from a CL as described in text. A representative field was selected containing a single plasma membrane fragment. Reconstitution was performed and photographed as described in Fig. 1 except that the reaction was initiated by the addition of buffer containing 44 μM free Ca^{2+}. (*A*) PM lawn. (*B*) RL formed by reconstitution of the lawn in *A*. (*C* and *D*) Same RL, 5 and 60 s, respectively, after addition of the Ca^{2+}-containing buffer. Bar, 10 μm.

number of CV aggregates. Nevertheless, RLs prepared with these CVs responded to Ca^{2+} in a manner identical to the RLs in Figs. 2 and 3 (data not shown). Upon neutralization to pH 6.8, CVs prepared by the pH 9.1 procedure also undergo time-dependent aggregation, thus, in order to limit CV–CV aggregation, and to maximize the number of monomeric CVs bound to RLs, we routinely bring the purified suspension of CVs to pH 6.8 just before applying them to PM lawns.

In other experiments, we observed that Ca^{2+}-triggered exocytosis in RLs was inhibited by NEM modification conditions similar to those required to inactivate CL and CSC (9, 11). We also determined that RLs cannot be produced with CVs prepared from NEM-inactivated CSC; with PM lawns prepared from NEM-treated eggs; or with chymotrypsin-treated PM lawns (Crabb, J. H., and R. C. Jackson, unpublished results). These observations indicate that RLs, like CLs and CSC, are sensitive to sulfhydryl modification and suggest that proteinaceous elements are required for RL formation.

Threshold Ca^{2+} Concentration Required for Exocytosis in RLs

The validity of any in vitro exocytotic system must be judged by how closely it reflects the in vivo free Ca^{2+} requirement, estimated to be 1–12 μM in the sea urchin egg (20, 21).

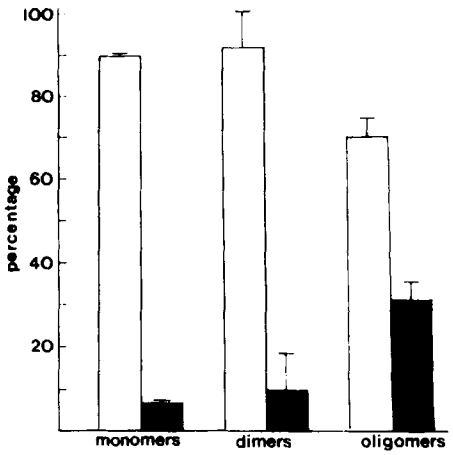

FIGURE 4 Isolated CVs do not exhibit calcium-dependent lysis. The percentage of purified CVs that remained intact (white bars) or were lysed by Ca^{2+} (black bars) was scored from micrographs of CVs immobilized on a bed of 1% agarose, taken before and after the free Ca^{2+} concentration was increased to 2 mM, as described in Materials and Methods. 3.8% of the monomeric CVs were apparently dislodged from the agarose surface during the Ca^{2+} addition. The contribution of these CVs is reflected in the total percentage (96.2%) of lysed + intact monomers. Error bars indicate SD of the mean (three separate CV preparations were tested).

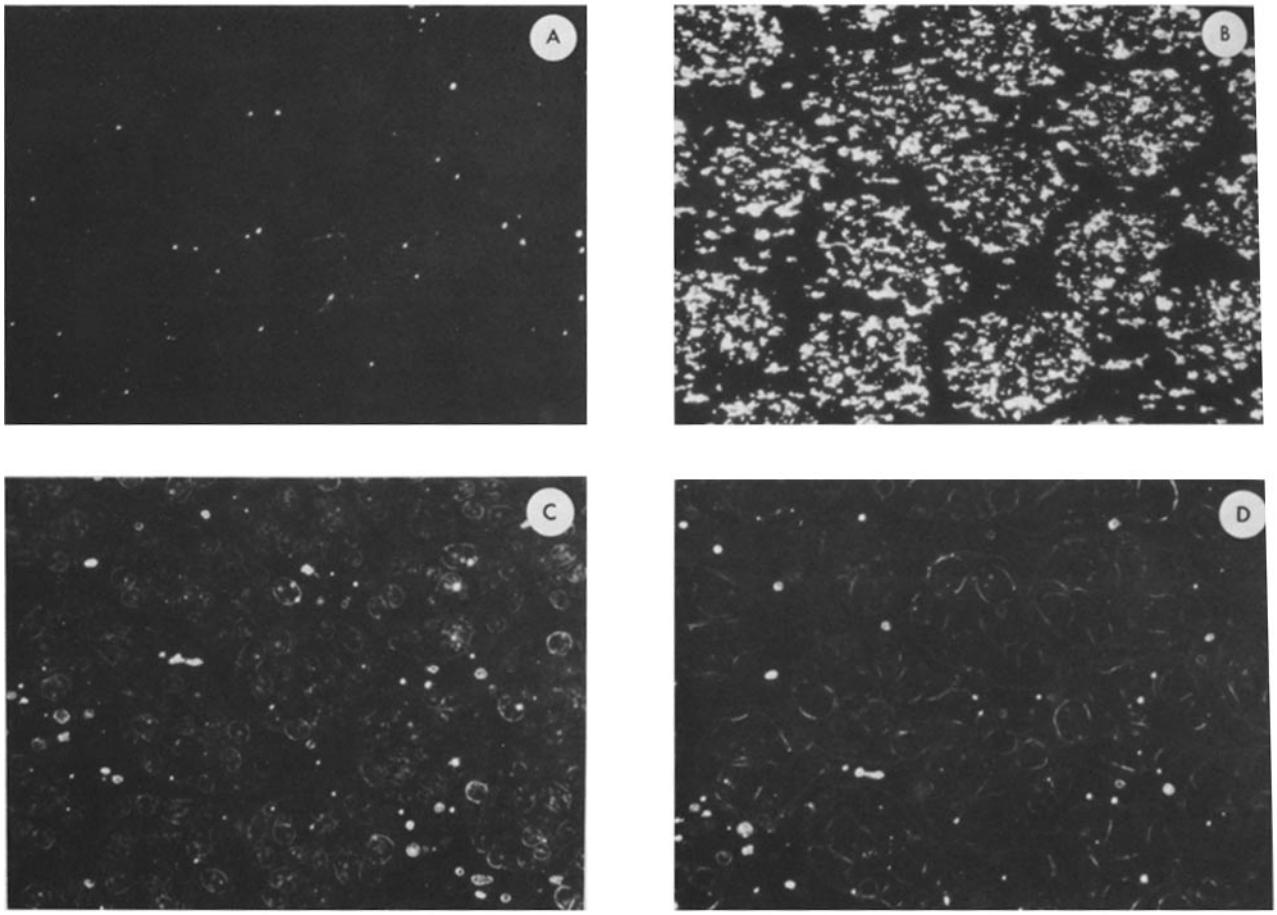

FIGURE 5 RL preparation and reaction viewed with darkfield optics. An RL was prepared and exocytosis was initiated as in Fig. 2, except that a low power field, corresponding to that used in the microphotometric assay, was chosen. (*A*) Plasma membrane lawn. (*B*) RL formed by reconstitution of the lawn in A with purified CVs. (*C* and *D*) Same lawn 5 and 30 s, respectively, after addition of buffer containing 2 mM free Ca^{2+}. × 300.

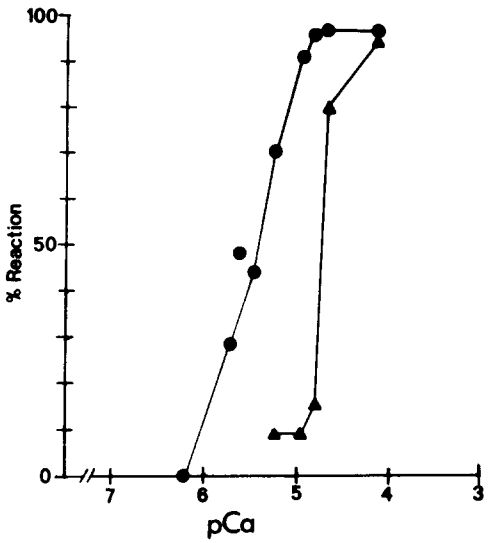

FIGURE 6 Calcium sensitivity of exocytosis in RLs and CSC lawns. RLs (▲) and CSC lawns (●) were prepared and assayed over a range of free Ca^{2+} concentrations with the microphotometric assay as described in Materials and Methods. Each point represents the extent of exocytosis on a separate lawn sample assayed at the indicated free Ca^{2+} concentration.

CL and CSC preparations, with half-maximal Ca^{2+} threshold values of 3.2 (7) and 3.5 μM (11), respectively, fulfill this criterion.

We determined the Ca^{2+} concentration required for half-maximal reaction in RLs by using a sensitive microphotometric assay based on light scattering from individual CVs (14a, 15). A PM lawn that had been completely stripped of CVs scattered little light (Fig. 5*A*). The individual PM fragments that comprise the lawn could not be clearly distinguished. However, when decorated with bound CVs (Fig 5*B*), the outlines of the individual CV–PM fragments were recognizable. Ca^{2+}-triggered CV–PM fusion (Fig. 5, *C* and *D*) resulted in the release of CV contents and a substantial decrease in the amount of scattered light.

In the microphotometric assay, the amount of light scattered by the CVs is quantitated by a photodiode assembly and recorded (14a, 15). Using this assay, we determined that the free Ca^{2+} concentration required for half-maximal reaction in RLs was 18.5 ± 2.2 μM (averages from three experiments similar to that shown in Fig. 6). For comparison, we prepared lawns from the same batch of CSC that had been used to prepare CVs for the RL. These CSC lawns were incubated for 10 min at room temperature to simulate reconstitution, and then assayed. The Ca^{2+} concentration required for half-maximal exocytosis in these CSC lawns was 5.7 ± 1.9 μM (Fig. 6).

The reason for the small increase in the Ca^{2+} threshold of RLs relative to CSC lawns is not known. Sasaki (22) has reported that KCl extraction of *Hemicentrotus pulcherrimus* CLs, but not *S. purpuratus* CLs (Sasaki and Epel [23]),

increases the Ca^{2+} threshold required for exocytosis and releases a proteinaceous Ca^{2+}-sensitizing factor. The relationship of this factor, if any, to the increased Ca^{2+} threshold of RLs is not clear. Indeed, in view of the potentially detrimental dissociative procedures used to prepare CV and PM lawns, and the inherent inefficiency of any reconstitution process, the similarity of the half-maximal Ca^{2+} thresholds of RLs, CLs, and CSC lawns is remarkable. It clearly suggests that the Ca^{2+}-triggered release reaction in RLs is the biochemical equivalent of exocytosis in CLs and CSC lawns.

Vectorial Transport of CV Contents Across the Plasma Membrane

We have shown above that purified CVs reassociate with PM lawns, and that the resultant reconstituted lawns have a Ca^{2+} threshold comparable to that required for exocytosis in intact eggs and cortical lawns. We have also demonstrated that reassociation of CVs with PM is a prerequisite for the observed reaction. However, to prove that the Ca^{2+}-triggered release of CV contents from RLs and CLs was the in vitro equivalent of exocytosis, it was necessary to demonstrate the vectorial transport of CV contents across the plasma membrane.

One method of demonstrating vectorial transport into a membranous compartment is through the use of a "protection" assay, in which the transported molecules are "protected" by the membrane against modification by exogenously added macromolecules. For instance, transport of nascent presecretory proteins into microsomal vesicles has been assayed by the resistance of the transported proteins to proteolytic degradation (24).

CV contents that have been transported across the plasma membrane of a CL or RL are delivered into a compartment that is bounded on one side by the egg plasma membrane and on the other by the extracellular vitelline layer through which the lawn is attached to the glass. This compartment is sealed around its circumference by association of the PM with the vitelline layer/polylysine-coated glass. This is an imperfect seal: CV contents spread beneath the membrane and can ultimately leak around the circumference. Nevertheless, it can be expected to provide a temporary barrier between transported CV contents and exogenously added probes.

The degree of protection afforded by this type of membrane configuration has been investigated by Kalish et al. (25). They showed that the extracellular face of erythrocyte membrane "lawns," prepared on polylysine-coated glass beads, was not accessible to two macromolecular probes: neuraminidase and galactose oxidase. In contrast, two membrane proteins with cytoplasmic surface domains, spectrin and band 3, were shown to be readily accessible to added proteases.

In principle, our immunofluorescent transport assay is quite simple: vectorially transported hyalin, a CV component, is incapable of combining with anti-hyalin antibodies unless the plasma membrane barrier that separates it from the medium has been disrupted by detergent. The polyclonal anti-hyalin serum we used specifically stained CVs in sectioned eggs (14, 26) and has been used to follow the cortical reaction in intact eggs (14).

Cortical exocytosis was initiated as described in Materials and Methods. Glutaraldehyde fixation was used to stop the spread of CV contents (it probably also decreased the permeability of the barrier formed by association of the plasma membrane with the polylysine-coated glass coverslip). The fixed lawn was subsequently incubated with an appropriately diluted anti-hyalin antiserum and fluorescein-conjugated goat anti-rabbit IgG, as described in Materials and Methods. Control lawns were permeabilized with detergent before incubation with antiserum.

The results of a typical vectorial transport experiment with a "sparse" CL is shown in Fig. 7. We used "sparse" CLs (CLs from which most CVs had been dislodged) in these experiments for two reasons: (*a*) it permitted the observation of individual CVs, eliminating the possibility of content release by the two-step process of CV–CV fusion, followed by lysis, and (*b*) preliminary experiments with whole CLs indicated that the large domes formed by the massive release of CV contents under the plasma membrane were unstable and occasionally lysed shortly after formation. This observation is in accord with a recent electron microscopic study of critical point–dried and rotary-shadowed CL samples (8). The small domes, formed by the vectorial discharge of single CVs, were more stable, and therefore easier to work with than the massive domes formed by the discharge of many CVs.

Fig. 7*A* shows that CVs released from a "sparse" CL, upon incubation with 44 μM Ca^{2+}, are released into a compartment that is not accessible to anti-hyalin antibodies. Detergent disruption of the membranous domes formed by fusion of the CVs with the plasma membrane permitted the released CV contents to combine with the anti-hyalin antibodies (Fig. 7*B*). The intensity of immunofluorescence in Fig. 7*B* is localized to the periphery of the remnants of domes formed by fusion of CVs with the plasma membrane. The reason for this distribution is not clear, however, we suspect that the phase-dense material displaying the immunofluorescent stain contains undispersed portions of the CV cores. As controls we confirmed that neither PM lawns nor CLs with intact CVs stained with the anti-hyalin serum (not shown). We also demonstrated that staining was eliminated when the anti-hyalin serum was not added, or when it was replaced by a non-immune rabbit serum (not shown). The results presented in Fig. 7 established the validity of the immunofluorescent transport assay, and confirmed electron microscopic observations (7, 8) suggesting that the release of CV contents from CLs is the in vitro equivalent of exocytosis.

We used the vectorial transport assay to investigate the release of CV contents from RLs. When RLs were bathed in 44 μM Ca^{2+}, CV contents were vectorially discharged across the plasma membrane into a compartment that was not accessible to anti-hyalin antibodies (Fig. 8*A*). As with CLs, detergent disruption of the domes formed by fusion of the CVs with the plasma membrane permitted the anti-hyalin antibodies to combine with CV contents (Fig. 8*B*). This result provided direct evidence for the functional reconstitution of in vitro exocytosis in the RL preparation.

DISCUSSION

The secretory pathway is comprised of a series of membrane-delimited compartments through which secretory, membrane, and lysosomal proteins are transported as they move from their site of synthesis in the endoplasmic reticulum to the cell surface, lysosome, or appropriate site. Transfer of proteins from one compartment to the next proceeds via the dissociation of membranous vesicles from the donor compartment, and the subsequent fusion of these vesicles with the appropri-

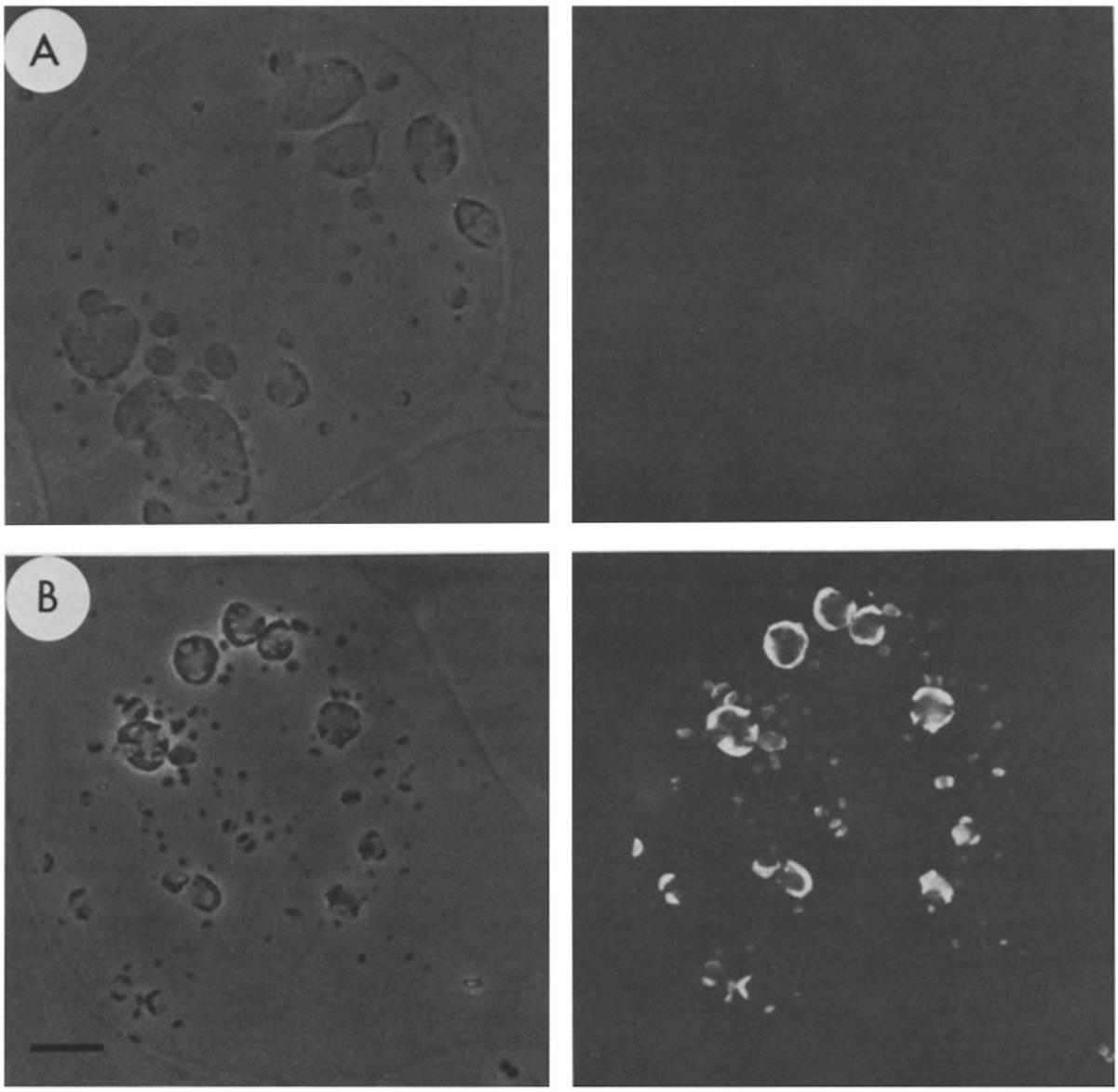

FIGURE 7 CV contents are vectorically transported across the plasma membrane in sparse CLs. CLs, sparsely decorated with CVs, were prepared, reacted with a buffer containing 44 μM free Ca^{2+}, and fixed as described in Materials and Methods. Anti-hyalin immunofluorescence of lawns with intact membranes (*A*) and of lawns whose membranes had been disrupted with Triton X-100 after glutaraldehyde fixation (*B*) was performed with CSC-absorbed anti-hyalin serum (at a dilution of 1:20) as described in Materials and Methods. The paired micrographs in *A* and *B* are phase-contrast (*left*) and immunofluorescent (*right*) images of the same field. Bar, 10 μm.

ate acceptor compartment (27).

The ability of the cell to deliver proteins to the correct compartment/membrane is thought to occur via the specific packaging of each class of protein into an appropriately targeted vesicle. It is for the reason that the various transfer steps in the secretory pathway, and membrane fission/fusion, in general, is currently the focus of intense interest.

Despite this interest, progress toward elucidation of the molecular biology of the various transfer steps in the secretory pathway has been slow. The primary impediment to progress in this area has been the absence of in vitro assays that faithfully reproduce the trafficking steps as they occur in the cell.

The final destination of secretory proteins is the extracellular space. Delivery of secretory proteins to this "compartment" proceeds by exocytosis: the fusion of secretory vesicles with the plasma membrane. We have taken advantage of the unique morphology of the sea urchin egg, and developed an in vitro assay for this step in the secretory pathway.

Our assay is based on the production of exocytotically competent reconstituted cortical lawns from isolated cortical secretory vesicles and plasma membrane. The evidence that the Ca^{2+}-triggered release of CV contents from RLs is the in vitro equivalent of exocytosis is fourfold. (*a*) Release of CV contents from RLs is morphologically identical to Ca^{2+}-triggered exocytosis in CLs (Figs. 2 and 3). (*b*) Reassociation of CVs with the plasma membrane is a prerequisite for the release reaction. Purified CVs alone are unresponsive even at millimolar concentrations of Ca^{2+} (Fig. 4). (*c*) The threshold Ca^{2+} concentration required for exocytosis in intact eggs, CLs, and CSC lawns is similar to that required for exocytosis in RLs (Fig. 6). (*d*) Ca^{2+} stimulation of RLs results in the

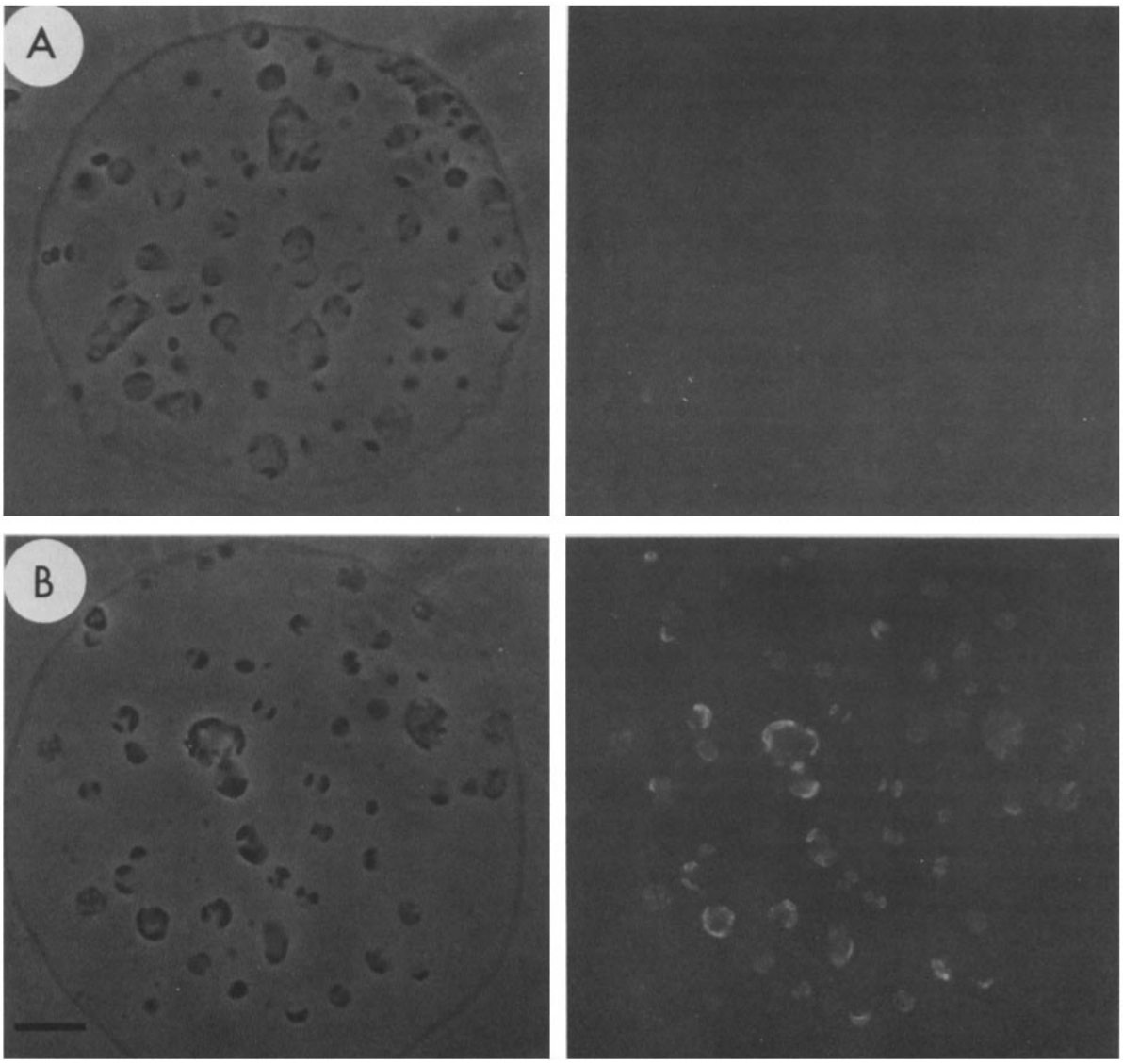

FIGURE 8 CV contents are vectorially transported across the plasma membrane in RLs. RLs were prepared, reacted with a buffer containing 44 μM free Ca^{2+}, and fixed as described in Materials and Methods. Anti-hyalin immunofluorescence was performed with CSC-absorbed anti-hyalin serum (at a dilution of 1:40) as described in Materials and Methods. (*A*) Paired phase-contrast and immunofluorescent images of a reconstituted cortical fragment with intact membranes. (*B*) Paired phase-contract and immunofluorescent images of a reconstituted cortical fragment with Triton X-100 disrupted membranes. Bar, 10 μm.

vectorial transport of hyalin, a CV content protein, across the plasma membrane (Fig. 8). In addition, the ability of NEM and proteases to block RL formation, and the ability of NEM to inhibit exocytosis in pre-formed RL (not shown) is consistent with the properties of CSC (11), and indicates that proteinaceous elements are required for CV binding and exocytosis.

The immunofluorescence technique that we developed for analysis of vectorial transport of CV contents across the plasma membrane has several advantages vis-a-vis electron microscopic analysis. It is more rapid than electron microscopy, and it relies on analysis of the transport of a specific CV content protein (hyalin). The use of CL from which most CVs have been dislodged permits individual CVs to be followed, and eliminates the complications introduced by the instability of the large membranous domes formed by the massive release of CV contents under the plasma membrane. Most importantly, with the immunofluorescent assay, vectorial transport can be analyzed over an entire field of cortical fragments, thus we have been able to determine that the contents of virtually all CVs in an RL or CL are vectorially discharged across the plasma membrane.

The reconstitution technology has two practical advantages. (*a*) The procedures for preparing CVs, PM lawns, and RLs are rapid and relatively simple. (*b*) The exocytotic release of CV contents from RLs can be easily quantitated (Figs. 5 and 6), and its kinetics analyzed with the microphotometric assay.

The development of quantitative assays for the reassociation of CVs with PM lawns is currently underway. This technology, in conjunction with development of specific reagents (e.g., anti-CV monoclonal antibodies) and conditions designed to reversibly disrupt CV binding, should facilitate identification of the molecules that comprise the CV–PM junction, and perhaps the molecule(s) responsible for CV–PM fusion as well.

The possibility that the technology we have developed with

the sea urchin egg may be of general utility for studying exocytosis in other secretory cell types has not escaped our notice. The threshold Ca^{2+} concentrations required for exocytosis, as well as sensitivity to various protein and membrane perturbants are nearly identical in every secretory cell type that has been examined. This suggests that the molecules responsible for Ca^{2+}-triggered exocytosis may be conserved, and perhaps even functionally interchangeable, from one cell type to another.

As a first step in this direction, we have formed heterologous RLs by combining CVs prepared from *S. purpuratus* with PM lawns derived from *Lytechinus pictus*. Preliminary results indicate that these heterologous RLs are exocytotically active. Whether the great majority of secretory cells in which a stable secretory vesicle–plasma membrane complex does not preexist can be used to produce homologous and/or heterologous RLs similar to those prepared with the sea urchin egg remains to be determined.

Though we have demonstrated the in vitro reconstitution of exocytosis, the extent to which this reaction approximates the in vivo situation remains to be determined. To gain insight into the in vitro reaction, we are currently studying the specificity of reassociation and fusion of CVs with other membranes (e.g., plasma membranes from secretory and nonsecretory cell types as well as planar phospholipid bilayers). These experiments should clarify the specificity of CV–PM interaction in vitro. However, we know so little about the specificity of this interaction in vivo that there is no way to ascertain unequivocally, on the basis of the in vitro specificity, how closely the reconstituted system reflects exocytosis in an intact cell. Hence, it will ultimately be necessary to identify the molecules responsible for exocytosis in vitro and to determine whether the same molecules are essential for exocytosis in vivo. Hopefully, the technology we have presented here will speed these studies and facilitate dissection of the molecular mechanism of exocytosis.

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