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Reassociation of Cortical Secretory Vesicles with Sea Urchin Egg Plasma Membrane: Assessment of Binding Specificity

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Summary. An assay has been developed for quantitating the reassociation of cortical secretory vesicles (CVs) with fragments of sea urchin egg plasma membrane attached to glass slides (PM lawns). Binding of *S. pupuratus* CVs to homologous PM lawns increased with time and CV concentration. The observation that CV binding was blocked by chymotrypsin digestion of the PM fragments suggested that a PM protein(s) is required for reassociation. The possibility that the extent of CV lysis that occurred during CV preparation (15.4 ± 3.8% as assessed by ovoperoxidase assay) influenced reassociation was investigated by determining the effect of CV content proteins (isolated as fertilization product) on binding. Various concentrations of fertilization product (up to equivalent amounts of fertilization product and CV protein) had no effect on CV binding. The specificity of binding was investigated by assessing the ability of CVs to bind to PM lawns prepared from human red blood cells, and by determining the ability of heterologous vesicles to bind to egg PM fragments. PM lawns from HRBCs did not support CV binding; however, PM lawns prepared from the eggs of several species of sea urchin did bind *S. pupuratus* CVs. Vesicles from a partially purified preparation of yolk platelets bound to egg PM lawns with low efficiency (1/7 that of CVs), but immunofluorescence analysis with an anti-hyalin monoclonal antibody demonstrated that 74 ± 9% of the bound vesicles were CVs that contaminated the yolk platelet preparation. Dioleoylphosphatidyl choline liposomes were also unable to bind to egg PM lawns. These results are consistent with hypothesis that CV binding to egg PM lawns is a specific, protein-mediated event.

Key Words sea urchin egg · exocytosis · plasma membrane · secretory vesicles

Introduction

In the sea urchin, fertilization initiates a rapid, synchronous exocytotic event that releases the contents of cortical secretory vesicles (CVs)[1] onto the surface of the fertilized egg. The proteins and acid mucopolysaccharides released from the CVs combine with the vitelline layer of the egg to form the fertilization envelope, a tough extracellular investment that establishes a permanent block to polyspermy and protects the developing embryo (Kay & Shapiro, 1985).

Despite the large size of the egg (approximately 80 μm in diameter), cortical exocytosis is rapid and proceeds nearly to completion (Whitaker & Steinhardt, 1985). Its rapidity is important for establishing a timely block to polyspermy. The fact that it proceeds nearly to completion is not surprising, since it occurs but once during the life of an urchin! The speed of cortical exocytosis is undoubtedly a consequence of the egg's unique morphology; the CVs of the mature egg are docked at the plasma membrane, poised for exocytosis (Anderson, 1970; Vacquier, 1975; Detering et al., 1977; Hylander & Summers, 1981). Exocytosis is initiated at the site of sperm-egg fusion and is propagated to the antipode of the egg (Moser, 1939; Rothschild & Swann, 1949) by a mechanism that involves the release of Ca^{2+} from a cortical reticulum (Sardet, 1984) by IP_3 (Clapper & Lee, 1985; Oberdorf, Head & Kaminer, 1986; Swann & Whitaker, 1986; Turner, Jaffe & Fein, 1986; Henson et al., 1988).

Fragments of egg cortex comprise an exocytotically competent complex, consisting of the vitelline layer, the plasma membrane, and attached CVs. Two different preparations of egg cortex are currently in use. The cell surface complex preparation (Detering et al., 1977) consists of a suspension of

[1] *Definitions and major abbreviations:* CL: Cortical lawn, a preparation consisting of fragments of sea urchin egg cortex attached via their extracytoplasmic surface to a poly-L-lysine coated glass slide or coverslip; CV: Cortical secretory vesicle; PM: Plasma membrane, the term PM fragment is used to designate one of the individual pieces of PM that constitute a PM lawn; PM lawn: Plasma membrane lawn, a preparation consisting of PM fragments attached via their extracytoplasmic surface to a poly-L-lysine coated glass slide or coverslip; and RL: Reconstituted cortical lawn, formed by reattachment of CVs to a sea urchin egg PM lawn.

egg cortex fragments, and is best suited for biochemical analysis. The cortical lawn, or CL, preparation (Vacquier, 1975) consists of an array of egg cortex fragments attached via their extracytoplasmic surface to a polycation-coated glass slide or coverslip, and is convenient for microscopic observation.

When bathed in buffers containing Ca^{2+} concentrations in the micromolar range, both cortex preparations undergo a reaction that results in disappearance of the CVs (Vacquier, 1975; Detering et al., 1977). In CL preparations, this reaction has been shown by electron microscopy (Whitaker & Baker, 1983; Chandler, 1984; Zimmerberg, Sardet & Epel, 1985) and immunofluorescence (Crabb & Jackson, 1985) to result in the vectorial discharge of CV contents across the PM.

Small quantities of egg PM can be conveniently prepared by removing the CVs from a cortical lawn preparation of egg cortex (Kopf, Moy & Vacquier, 1982; Crabb & Jackson, 1985). These PM preparations, known too as PM lawns, consist of a planar array of PM fragments affixed via their extracytoplasmic surface to the polycation-coated glass support. Previously, we have shown (Crabb & Jackson, 1985) that purified CVs are capable of reassociating with the fragments of egg PM that comprise a PM lawn. The resultant preparation, which consists of an array of PM fragments with reattached CVs, is known as a reconstituted lawn (RL). In response to Ca^{2+}, RLs undergo a morphological change which appears by several criteria to be equivalent to the in vitro exocytosis reaction of CLs (Crabb & Jackson, 1985). These results suggest that reconstitution correctly reassembles a functional CV-PM junction. Additional insight into this question can be obtained through investigation of the specificity of CV-PM reassociation. To this end we have developed a quantitative assay for CV binding, and have used it to assess the specificity of CV-PM reassociation. A preliminary report of a portion of this work has been published as part of the proceedings of a conference (Crabb, Modern & Jackson, 1987).

Materials and Methods

Materials

Strongylocentrotus purpuratus and *Lytechinus pictus* were maintained at 9–12 and 12–15°C, respectively, in a refrigerated aquarium containing Instant Ocean seawater from Aquarium Systems (Mentor, OH). The following reagents were purchased from Sigma Chemical (St. Louis, MO): TPCK-trypsin, TLCK-chymotrypsin, DFP-chymotrypsin, diisopropylfluorophoshphate (DFP), soybean trypsin inhibitor (SBTI), ovoinhibitor, poly-L-lysine (molecular weight 2×10^5), HEPES, PIPES, EGTA, DL-dithiothreitol, goat serum, guaiacol, sodium deoxycholate, bovine serum albumin, sucrose, A23187, FITC-rabbit anti-mouse IgG (whole molecule), and FITC-goat anti-rabbit IgG (whole molecule).[2] $CHCl_3$, KCl, NH_4Cl, $MgCl_2$, NaH_2PO_4, NaCl, and H_2O_2 were from Fisher Scientific. Dioleoylphosphatidylcholine was a gift from Dr. G. Lienhard (Dartmouth Medical School). Rabbit anti-spectrin IgG was prepared by protein A-Sepharose chromatography (Goding, 1976) of anti-spectrin serum (a generous gift from Dr. P. Agre of Johns Hopkins School of Medicine). The control rabbit IgG fraction was prepared by protein A Sepharose (Pharmacia, Piscataway, NJ) chromatography of non-immmune rabbit serum. The mouse IgM monoclonal antibody to human blood group A antigen was a gift from Drs. J. Allard and G. Lienhard (Dartmouth Medical School). The control mouse IgM monoclonal was a gift from Dr. M. Fanger (Dartmouth Medical School). The mouse IgA monoclonal to hyalin was prepared by Dr. Carol Vater in this laboratory (Vater & Jackson, 1989*a*).

Preparation of CVs and PM Lawns

Urchins were spawned by intraceolomic injection of 0.5 M KCl; eggs were collected into ice-cold seawater. Eggs were filtered through a 125- (*S. purpuratus*) or 210-μm (*L. pictus*) mesh nylon screen (Small Parts, Miami, FL), and washed by three cycles of centrifugation and resuspension in 0.45-μm-filtered seawater. Eggs were either dejellied immediately with an EGTA-containing buffer and used to prepare egg cell surface complex, or maintained on ice as a 20% (vol/vol) suspension in seawater containing 10 μg/ml of SBTI, and subsequently dejellied and used for preparation of cortical lawns and PM lawns. Eggs could be stored in seawater containing 10 μg/ml SBTI for several hours and still adhere tightly to polylysine-coated slides; eggs stored without SBTI gave more variable results.

Cell surface complex was prepared as described by Jackson, Ward and Haggerty (1985) except that 10 μg/ml SBTI was added to the homogenization and resuspension buffers, and 1 μg/ml SBTI was added to all wash buffers. CVs were prepared from cell surface complex as described by Crabb and Jackson (1985), with the exception that the CV dissociation buffer ($KEAS_{10}$) was reformulated to contain 600 mM KCl, 5 mM EGTA, 50 mM NH_4Cl, 10 μg/ml SBTI, pH 9.1.

Cortical lawns and PM lawns were prepared as previously described (Crabb & Jackson, 1985) except that eggs were sheared with PKME buffer (50 mM PIPES, 425 mM KCl, 10 mM $MgCl_2$, 10 mM EGTA, pH 6.8) containing 1 μg/ml SBTI, and stored in PKME containing 10 μg/ml SBTI. For the chymotryptic digestion experiment (*see* Fig. 3) PM lawns were prepared without SBTI.

PM Lawns from Human Red Blood Cells

Red cell lawns, RBC-ghost lawns, and RBC-PM lawns were prepared as follows: human type A blood from healthy human volunteers was drawn into CPDA1 (2 g dextrose, 1.66 g sodium

[2] *Other abbreviations:* DIC: differential interference contrast; FITC: fluorescein isothiocyanate; HEPES: N-2-hydroxyethyl-N′-2-ethanesulfonic acid; HRBC: human red blood cell; PBS: phosphate-buffered saline; PIPES: piperazine-N,N′-*bis*[2-ethanesulfonic acid]; and SBTI: soybean trypsin inhibitor.

citrate, 206 mg citric acid, 140 mg NaH_2PO_4, 17.3 mg adenine in 63 ml). Blood was centrifuged at 1000 × *g* for 5 min, and the buffy coat was removed by aspiration. The pellet of red cells was resuspended in 140 mM NaCl, 20 mM HEPES, pH 7.4, washed four times with this buffer, and twice with SH buffer (280 mM sucrose, 20 mM HEPES, pH 7.4). The final red cell pellet was resuspended to 50% (vol/vol) in SH buffer. RBC-PM lawns were prepared on poly-L-lysine coated glass slide chambers by a modification of the procedure used to prepare egg PM lawns (Crabb & Jackson, 1985). A slurry of RBCs was applied to the center of a poly-L-lysine coated slide and allowed to attach for 5 min. Unattached cells were gently rinsed away with a stream of SH buffer containing 1 μg/ml SBTI. The resultant red cell lawn consists of intact red cells attached to the glass via their extracellular surfaces. RBC-PM lawns were prepared by shearing red cell lawns with a jet of SH buffer containing 1 μg/ml of SBTI delivered from a 50-ml syringe fitted with a 22-gauge needle. For reconstitution experiments, RBC-PM lawns were stored for brief periods of time in SH buffer containing 10 μg/ml SBTI, and washed into PKME buffer containing 10 μg/ml SBTI just prior to adding CVs. RBC-ghost lawns were prepared by hypotonic lysis of the intact cells of a red cell lawn with 5 mM NaH_2PO_4, pH 8.0.

For the DIC micrographs (*see* Fig. 5), red cell lawns, RBC-ghost lawns, and RBC-PM lawns (prepared on coverslips) were prepared as described above and rinsed into 140 mM NaCl, 20 mM HEPES, pH 7.4. DIC photographs were taken on Pan-X film (Eastman Kodak) using a Nikon Optiphot microscope and a 40× plan-apochromat oil immersion objective. For immunofluorescence (*see* Fig. 6), RBC-ghost lawns and RBC-PM lawns (on coverslips) were rinsed with 100 mM NaCl, 50 mM HEPES, pH 7.4, fixed for 10 min with 1% glutaraldehyde in 100 mM NaCl, 50 mM HEPES, pH 7.4, and washed twice for 5 min in 150-ml PBS (150 mM NaCl, 5 mM NaH_2PO_4, pH 7.4). After a 30-min preincubation with 5% normal goat serum in PBS, the fixed lawns were treated for 60 min with first antibody (25 μg/ml of an anti-blood group A specific mouse IgM monoclonal; 25 μg/ml of a control mouse IgM; 25 μg/ml of rabbit anti-spectrin IgG or 25 μg/ml of nonimmune rabbit IgG) in 5% normal goat serum/PBS. Unbound antibody was removed by washing twice for 10 min with PBS, and the samples were treated for 30 min in the dark with FITC conjugated second antibody (1/500 dilution of FITC-goat anti-mouse IgG in 5% normal goat serum/PBS or 1/500 dilution of FITC-goat anti-rabbit IgG in 5% normal goat serum/PBS). The samples were washed twice for 5 min in PBS to remove the fluorescent antibody, and mounted on chamber slides. Immunofluorescence photographs were taken on Ilford HP5 film (push processed to ASA 3200) using a Zeiss universal microscope and a 63× plan-apochromat oil immersion objective.

Yolk Platelets and Liposomes

All preparative procedures were conducted on ice or at 4°C unless otherwise indicated. Yolk platelets were prepared by a modification of the procedure described by Ii et al. (1978). Washed, dejellied *S. purpuratus* eggs were resuspended at 10% (vol/vol) in 50 mM PIPES, 425 mM KCl, 25 mM EGTA, 10 μg/ml SBTI, pH 6.8 and homogenized as for the preparation of CVs. The homogenate was centrifuged twice for 3 min at 250 × *g* to remove intact eggs and fragments of egg cortex. The supernatant was removed, transferred to 30-ml Corex tubes, and centrifuged at 2600 × *g* for 10 min in a swinging bucket rotor. The pellet of yolk platelets was washed 1× by resuspension in 50 mM PIPES, 425 mM KCl, 25 mM EGTA, 1 μg/ml SBTI, and centrifugation. The pellet of yolk platelets was resuspended in $KEAS_{10}$ buffer and adjusted to A_{400} = 10.0 just prior to use in reconstitution assays.

To prepare liposomes, 4.0 mg of dioleoylphosphatidylcholine (in $CHCl_3$) was evaporated into a small test tube with a stream of nitrogen. Residual traces of $CHCl_3$ were removed by lyophylization for 1 hr. $KEAS_{10}$ buffer (1.0 ml) was added to the tube and the dried phospholipid was allowed 5 min to hydrate. The tube was vortexed vigorously for 1 min, and A_{400} determined.

Fertilization Product

The method used to prepare fertilization product is based on the procedures described by Weidman and Kay (1986). Eggs were washed 2× in seawater, egg jelly removed, and the vitelline layer disrupted by incubation (6 min) with seawater containing 20 mM dithiothreitol, pH 9.4. After thorough washing with seawater, the eggs were resuspended in 600 mM KCl, 5 mM EGTA, pH 8.0, pelleted, and resuspended at 25% (vol/vol) in the same buffer containing 50 μg/ml SBTI and 1 mM diisopropylfluorophosphate. Cortical exocytosis was initiated by adding Ca^{2+} ionophore A23187 (10 mM stock in dimethyl sulfoxide) to a final concentration of 25 μM. After 5 min at room temperature, the eggs were centrifuged at 250 × *g* for 3 min. The supernatant was collected and centrifuged at 8000 × *g* for 10 min. The supernatant, containing the fertilization product, was clear and somewhat viscous. Prior to use in reconstitution experiments sufficient NH_4Cl (solid) was added to the fertilization product to achieve an NH_4Cl concentration of 50 mM, and the pH was adjusted to 9.1 with KOH.

CV Binding Assay

The procedure that we have developed for quantitation of CV binding is based on the ability of CVs to scatter light in the dark field microscope. Light scattering analysis was first introduced by Zimmerberg et al. (1985) as a means of determining percent exocytosis in cortical lawn preparations. It has also been used, by us (Crabb & Jackson, 1985) and others (Whalley & Whitaker, 1988) to determine the percent exocytosis in RL preparations. However, determination of percent exocytosis differs from quantitation of CV binding in one important respect. Percent exocytosis is an intrinsic measure (CVs reacted/total CVs) that can be obtained from analysis of a single sample without reference to other samples. For CV binding, the ability to compare binding of one sample to that of another, under different circumstances (e.g., at different CV concentrations), is essential. Thus, for a binding assay, it was necessary to prepare uniform PM lawns, and to establish uniform reassociation conditions.

To this end, three modifications were introduced in the RL preparation procedure (Crabb & Jackson, 1985): (*i*) PM lawns were prepared from a concentrated slurry of dejellied eggs (>25% vol/vol) so that each PM fragment was closely abutted by its neighbors. This proved to be the most convienient method of ensuring uniformity in the PM lawn preparation. (*ii*) Reconstitutions were conducted in a slide chamber of defined depth, so that the PM lawn was covered with a uniform depth of CV-containing suspension. (*iii*) The procedure for removing unbound CVs was standardized, so that differential shear forces did not skew the results.

The details of the RL preparation procedure are as follows: aliquots (600–1000 μl) of a CV suspension were brought to pH

6.8 by the addition of 1.0 M PIPES, pH 6.1, and 150-μl aliquots were drawn (with a 2 × 2 cm wick of filter paper) into slide chambers containing PM lawns. This process was repeated until CV suspension was added to all of the chambers to be used in a particular experiment. The design of the slide chambers (Crabb & Jackson, 1985) ensured that PM fragments in all samples were covered with a uniform depth (equivalent to the thickness of a #1 coverslip: 0.13 to 0.16 mm) of CV suspension. At the end of the indicated incubation period (15 min, unless otherwise indicated), the coverslip was removed from the chamber and unbound CVs washed away by dipping the slides five times into each of two 100-ml beakers of PKME buffer containing 1 μg/ml SBTI. Degassed PKME containing 10 μg/ml SBTI was added to the samples. A coverslip was placed on the chamber, and the samples were stored in a humid chamber until CV binding was measured. PM lawns and CVs were used within 40 min of preparation.

CV binding was measured by quantitating the light scattered from bound CVs by dark field microscopy. The bottom of the slide chamber and the top of the coverslip were carefully cleaned with a cotton tipped applicator that had been dipped in water to remove any spots of crystallized salt (from buffer evaporation) that may have accumulated on these surfaces. Scattered light was quantitated with a photodiode apparatus (Crabb & Jackson, 1986) and displayed on a potentiometric chart recorder. With our apparatus, the spot from which CV binding is recorded has an area of 98,000 μm^2 (equivalent to approximately 22 egg PM fragments with an average diameter of 75 μm). Within this spot, the signal was shown to be directly proportional to surface area. This proportionality was determined at low light intensity with bright field optics. The size of the field was varied with the field diaphragm of the microscope, and its area determined with the aid of a stage micrometer. A plot of the area of the field *vs.* the output of the photodiode is linear up to a field diameter of 354 μm. Transient fluctuations in the line voltage were eliminated with a voltage regulator (True Power-500, Acme Electric). For the experiments shown in Fig.1 a series of scattering standards was constructed. Microscope slides were scored with a diamond stylus. Two scores of varying depths were made on each slide, and a second slide was glued over the scored surface of the first. The extent of scatter from each standard was optimized by centering the point at which the two scores intersected in the microscope field. Day-to-day variation in the standard curve amounted to ±50 mV.

The ability of RBC-PM lawns to support the binding of CVs, and the ability of yolk platelets and liposomes to bind to egg PM lawns were assayed exactly as described above for CV binding.

Protease Digestions

Chymotryptic digestion of egg PM lawns was conducted at room temperature for 5 min. The indicated concentration of chymotrypsin or DFP-inactivated chymotrypsin in PKME buffer was drawn into a microscope slide chamber containing a PM lawn. The reaction was stopped by drawing PKME buffer containing 10 μg/ml of SBTI and 10 μg/ml of ovoinhibitor into the slide chamber. The ability of CVs to bind to chymotrypsin-treated PM lawns was determined as described above, except that all buffers contained ovoinhibitor at a concentration equivalent to that of SBTI.

The effect of tryptic digestion of CVs was determined as follows: sufficient trypsin or DFP-inactivated trypsin (3 mg/ml stock solution in 1 mM HCl) was added to an aliquot of CVs to obtain the desired final trypsin or DFP-trypsin concentrations. After 20-min incubation on ice, digestion was stopped by adding SBTI to a final concentration of 400 μg/ml from a 10-mg/ml stock, and the ability of the CVs to bind to egg PM lawns was determined as described above. In separate experiments it was shown that the amount of SBTI and ovoinhibitor used completely inhibited the highest concentration of trypsin and chymotrypsin used in these protease digestion experiments.

Other Procedures

Protein was determined by the method of Bradford (1976), using bovine serum albumin as a standard. The extent of CV lysis that occurred during CV preparation and formation of RLs was determined as follows: CVs (1.5 ml) were prepared as described above, brought to pH 6.8 with 1.0 M PIPES, and incubated for 15 min at room temperature. An aliquot of this suspension was layered over a 2.0-ml cushion of 15% (wt/vol) sucrose in KEA buffer and centrifuged at 16,000 × *g* for 10 min in a swinging bucket rotor. A 0.75-ml aliquot of the supernatant was removed and its ovoperoxidase activity compared to that of the original CV suspension. Ovoperoxidase activity was determined from initial rates, essentially as described by Diets et al. (1984). The final concentrations of reagents in the assay were 48 mM HEPES, 0.096% sodium deoxycholate (to lyse intact CVs), 17.28 mM guiacol, 12 mM KCl, 0.1 mM EGTA, 1.0 mM NH_4Cl, 0.2 μg/ml SBTI, 0.3 mM H_2O_2, pH 7.5.

Results

CV Binding Assay

The results presented in Fig. 1 show that binding of *S. purpuratus* CVs to a homologous *S. purpuratus* PM lawn increased as a function of incubation time. Binding curves obtained on two different days, with different batches of eggs, are shown. Both curves have the same shape, but differ somewhat in the magnitude of CV binding. These representative curves illustrate the extent of variation that we have observed with different preparations of CVs and PM lawns. At present the cause of this variation is not known, but it would appear to be the result of differences in the CVs and PM fragments themselves, since scattering standards were used to calibrate the instrument prior to both experiments, and since the inclusion of additional protease inhibitors (leupeptin, pepstatin and aprotinin) did not increase binding.

Both curves showed signs of approaching a plateau by 80 min. For comparison, the amount of light scattered from cortical lawns prepared from the same eggs that were used to prepare CVs and PM lawns for the reassociation curves is also presented. Although the maximal amount of binding to PM lawns approaches that recorded from cortical lawn samples, the CVs associated with the egg PM fragments were observed to be aggregated at the later

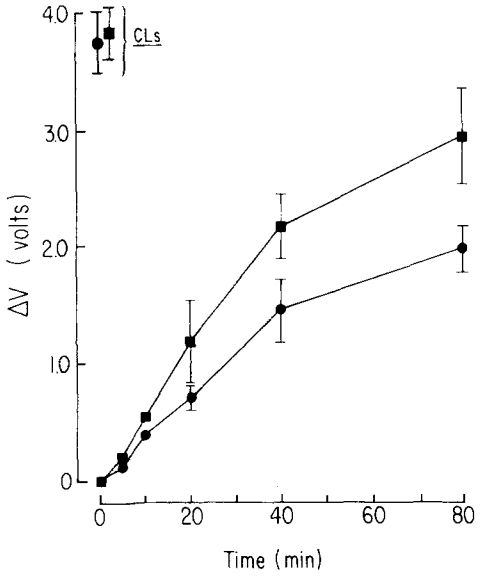

Fig. 1. Binding of CVs to egg PM lawns increases with time of incubation. *S. purpuratus* CVs (A_{400} = 10) were incubated with *S. purpuratus* PM lawns for the indicated time at room temperature. Binding was quantitated as described in Materials and Methods. Each data point represents the mean ± SD of triplicate samples. In this and all subsequent figures, data points without error bars indicate that the SD was less than the size of the data point. The two curves presented in this figure are results obtained with different preparations, and are representative of five similar experiments

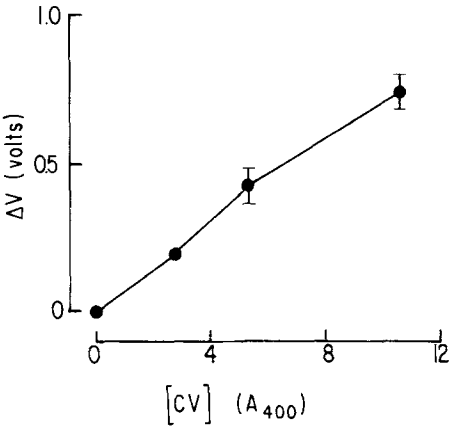

Fig. 2. Binding is a linear function of CV concentration. The indicated concentrations of CVs were incubated with PM lawns for 10 min, and binding was quantitated as described in Materials and Methods. Each data point is the mean ± SD of triplicate samples. Results are representative of seven similar experiments

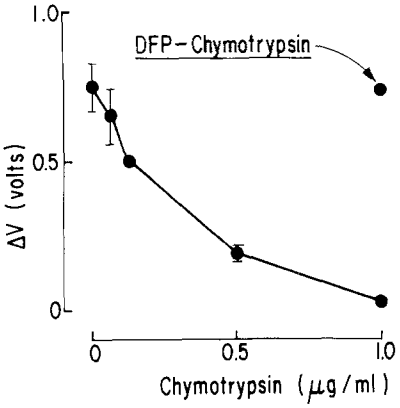

Fig. 3. Chymotrypsin treatment of PM lawns inhibits CV binding. PM lawns were treated for 5 min at room temperature with the indicated concentration of TLCK-chymotrypsin or DFP-inactivated chymotrypsin. Digestion was terminated by washing the lawns in a buffer containing 10 μg/ml each of ovoinhibitor and SBTI. Chymotrypsin-treated lawns were incubated with CVs (A_{400} = 13.9) for 15 min and binding was quantitated as described in Materials and Methods. Results are the mean ± range of duplicate samples, and are representative of five similar experiments

time points. Hence, the fact that the magnitude of CV binding approaches that seen in CLs must be interpreted with caution.

Figure 2 shows that CV binding to PM lawns was a linear function of CV concentration. In this experiment, binding was assessed at t = 10 min, so that CV aggregation would not be a problem. As a result, plateau levels of CV binding were not reached. Taken together, the data presented in Figs. 1 and 2 show that CV binding can be quantitated by the described light scattering procedure.

Proteolytic Digestion of PM Blocks CV Binding

If the reassociation of CVs with egg PM is a specific event, it is likely to involve the interaction of proteins located on the cytoplasmic surfaces of the CVs and PM. We addressed this question by determining the effect of proteolytic digestion of CVs and PM lawns on binding.

In one set of experiments, egg PM lawns were treated with various concentrations of chymotrypsin and their ability to bind CVs was assessed. Chymotrypsin was chosen for these experiments, because it can not hydrolyze the poly-L-lysine substrate used to attach PM fragments to the glass slide, and thus, under the conditions used, did not detach PM fragments from their glass support (*data not shown*). The results of these experiments (Fig. 3) clearly demonstrate that chymotryptic digestion of the cytoplasmic surface of the PM fragments prevented CV binding. Treatment of PM fragments with DFP-inactivated chymotrypsin did not inhibit binding, indicating that enzymatic activity is re-

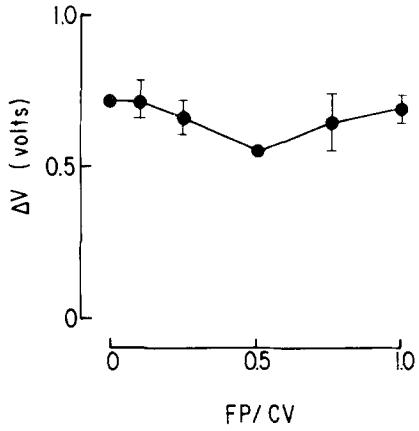

Fig. 4. CV binding is not affected by the addition of fertilization product. Fertilization product (FP) was prepared by ionophore A23187 (25 μM) treatment of vitelline layer-free dejellied eggs as described in Materials and Methods. CVs (A_{400} = 14.0) were supplemented with sufficient fertilization product and buffer to achieve the indicated FP/CV (protein/protein) ratios and a CV concentration of A_{400} = 5.0. Binding was determined as described in Materials and Methods. Results are the mean ± SD of triplicate samples, and are representative of four similar experiments

quired for inhibition of binding. This experiment suggests that a proteinaceous element(s) on the cytoplasmic surfaces of the PM is required for reassociation of purified CVs with PM lawns.

The possibility that proteinaceous surface elements of the CV are also required for CV-PM reassociation was tested by treating CVs with trypsin. Tryptic digestion was stopped with SBTI, and CV binding determined as described in Materials and Methods. Digestion of CVs (A_{400} = 10.9) with 50–100 μg/ml of trypsin (20 min at 4°C) decreased CV binding to approximately 25% of the control, but also resulted in CV aggregation; thus, it was not clear whether the decreased binding was a direct result of tryptic digestion or a secondary result of CV aggregation (*data not shown*).

CV Lysis Is Not a Factor

In analyzing CV-PM reassociation it is important to consider the possibility that proteins and mucopolysaccharides released by CV lysis during CV preparation and RL formation may positively or negatively enhance CV-PM reassociation. This potential source of error was addressed by determining the extent of CV lysis, and by analyzing the effect of CV contents on the reassociation reaction.

Using ovoperoxidase as a marker enzyme for CV content proteins, the extent of CV lysis that occurred during CV preparation and mock (15 min) RL formation was determined to be 15.4 ± 3.8% (mean ± SD from three CV preparations with six assay points per preparation). If this level of lysis was affecting the CV-PM reassociation reaction, it is probable that a greater extent of lysis would have even more pronounced effects. Thus, in order to assess the potential effect of CV contents on RL formation, the CV suspension used for RL formation was supplemented with increasing concentrations of CV contents.

As a concentrated source of CV contents for these experiments we collected fertilization product, which is the exudate of cortical exocytosis. CV suspensions were supplemented with increasing concentrations of freshly prepared fertilization product up to a CV/fertilization product ratio (protein/protein) of 1/1, and assayed for RL formation. The results (Fig. 4) of this experiment showed that even high concentrations of fertilization product had no effect on CV binding. Another experiment in which hypotonically lysed CVs rather than fertilization product was used as a source of CV contents gave similar results (*data not shown*). Since the exceedingly high amounts of CV contents used in these experiments had no effect on CV-PM reassociation, it can be concluded that the small percentage of CV lysis that inevitably occurred during CV preparation and RL formation probably has no effect on CV-PM reassociation.

Specificity of CV Binding: Binding to Heterologous PMs

If CV binding to egg PM lawns is mediated by a specific protein, heterologous PM fragments might not support CV binding. As a heterologous source of PM we chose to use human red blood cells. The red cell contains no secretory vesicles; thus, its membrane was considered to be unlikely to support CV binding. Furthermore, it was clear from the previous work of Jacobson and Branton (1977) that red cells bind tightly to cationic surfaces; hence, it seemed likely that it would be possible to prepare RBC-PM lawns, comparable to those prepared from sea urchin eggs.

Washed RBCs were attached to polylysine-coated slides in a low ionic strength sucrose-based buffer identical to that used by Jacobson and Branton (1977) for attachment of RBCs to glass beads. Confluent lawns of RBC-PM fragments could be formed by shearing the attached RBCs with a jet of the same buffer forcefully delivered from a 50-ml syringe fitted with a 22-gauge needle. Lawn formation could be conveniently followed by visually observing the clearing of the red color of the RBC

lawn. Compared to the preparation of egg-PM lawns, the preparation of RBC-PM lawns required the use of much greater shear force. This difference probably results from the much smaller size of RBCs.

DIC micrographs of an RBC lawn prior to shearing, a ghost lawn, prepared by hypotonic lysis of an RBC lawn, and an RBC-PM lawn are shown in Fig. 5, panels *A*, *B*, and *C*, respectively. The RBCs in Fig. 5*A* do not have the traditional bioconcave disk shape or HRBCs; rather, they are rounded and somewhat crenated. The shape of the attached RBCs could result from binding of the cells to poly-lysine-coated glass, or it may be due to the fact that we have not provided the cells with a source of metabolic energy (glucose). We have not attempted to investigate this phenomenon further.

Hypotonic lysis of the RBCs produced a ghost lawn (Fig. 5*B*), which differed in appearance from the RBC-PM lawn (Fig. 5*C*). Several ghost fragments contained what appeared to be membrane folds, which presumably occur when the larger unattached portion of the membrane collapses onto the attached portion. In other instances, the unattached portion of the one ghost could be seen to have collapsed on top of another ghost. The PM lawn (Fig. 5*C*) was more regular in appearance. The fragments contained no folds of membrane, and individual fragments did not lie on top of each other. The edges of each of the PM fragments, which are apparently comprised of unsheared flaps of PM, were more uniform and distinct than the edges of the ghost fragments in Fig. 5*B*. The small dimples that are visible on some of the PM fragments in Fig. 5*C* could possibly be due to small pockets of buffer trapped under the membrane, but this has not been further investigated.

Although the microscopic images of RBC-PM lawns and ghost lawns strongly suggested that RBC-PM lawns were comprised of a single layer of RBC-PM oriented with its cytoplasmic face available to the medium, we confirmed this topology by immunofluorescence analysis of the preparations. Ghost lawns and RBC-PM lawns were probed with antibodies directed to cell surface or cytoplasmic surface antigens (the blood group A antigen and spectrin, respectively), as described in Materials and Methods. The results (Fig. 6) of this experiment demonstrate that RBC-PM lawns have the expected topology. With the RBC-PM preparation, the monoclonal antibody to the human blood group A antigen stained only the small unsheared flaps of membrane at the periphery of each PM fragment (Fig. 6*A*). With the ghost lawn the anti-blood group A monoclonal antibody stained the entire surface of each of the ghost fragments (Fig. 6*B*). In contrast,

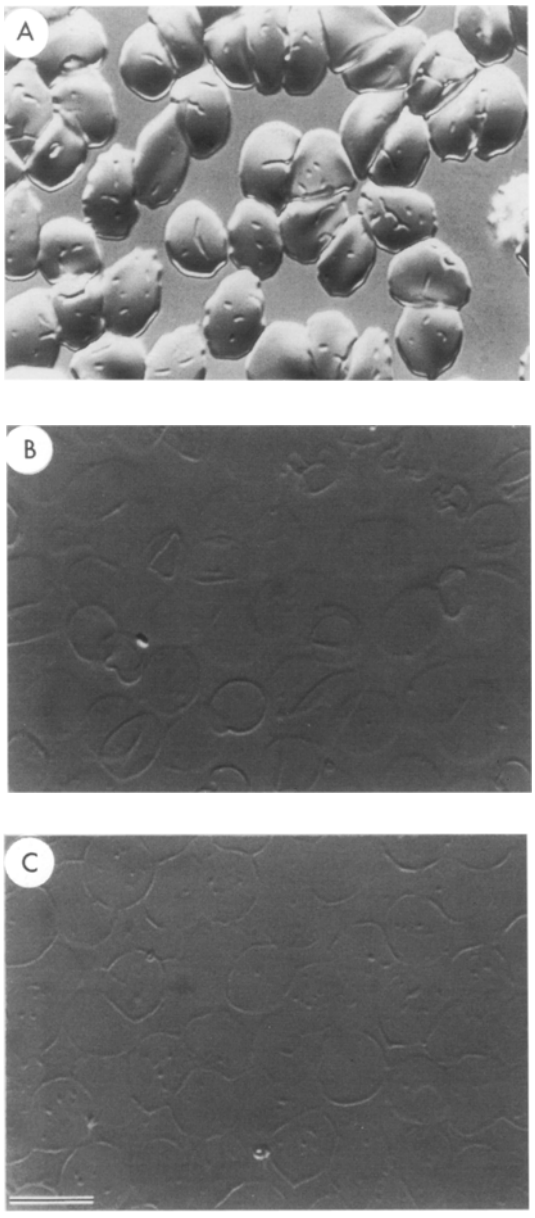

Fig. 5. DIC images of RBC lawn preparations. RBC lawns (*A*), RBC-ghost lawns (*B*), and RBC-PM lawns (*C*) were prepared as described in Materials and Methods, and photographed with a microscope equipped with differential interference optics. Bar is 10 μM in length

antibodies to spectrin, the major component of the membrane attached cytoskeleton of the RBC, stained the entire surface of each of the PM fragments in a RBC-PM lawn preparation (Fig. 6*C*). These immunofluorescence experiments established that the cytoplasmic face of the individual PM fragments in a RBC-PM is available to the medium. The results of our immunofluorescence analysis agree with electron microscopic observations of this preparation (Ursitti et al., 1988).

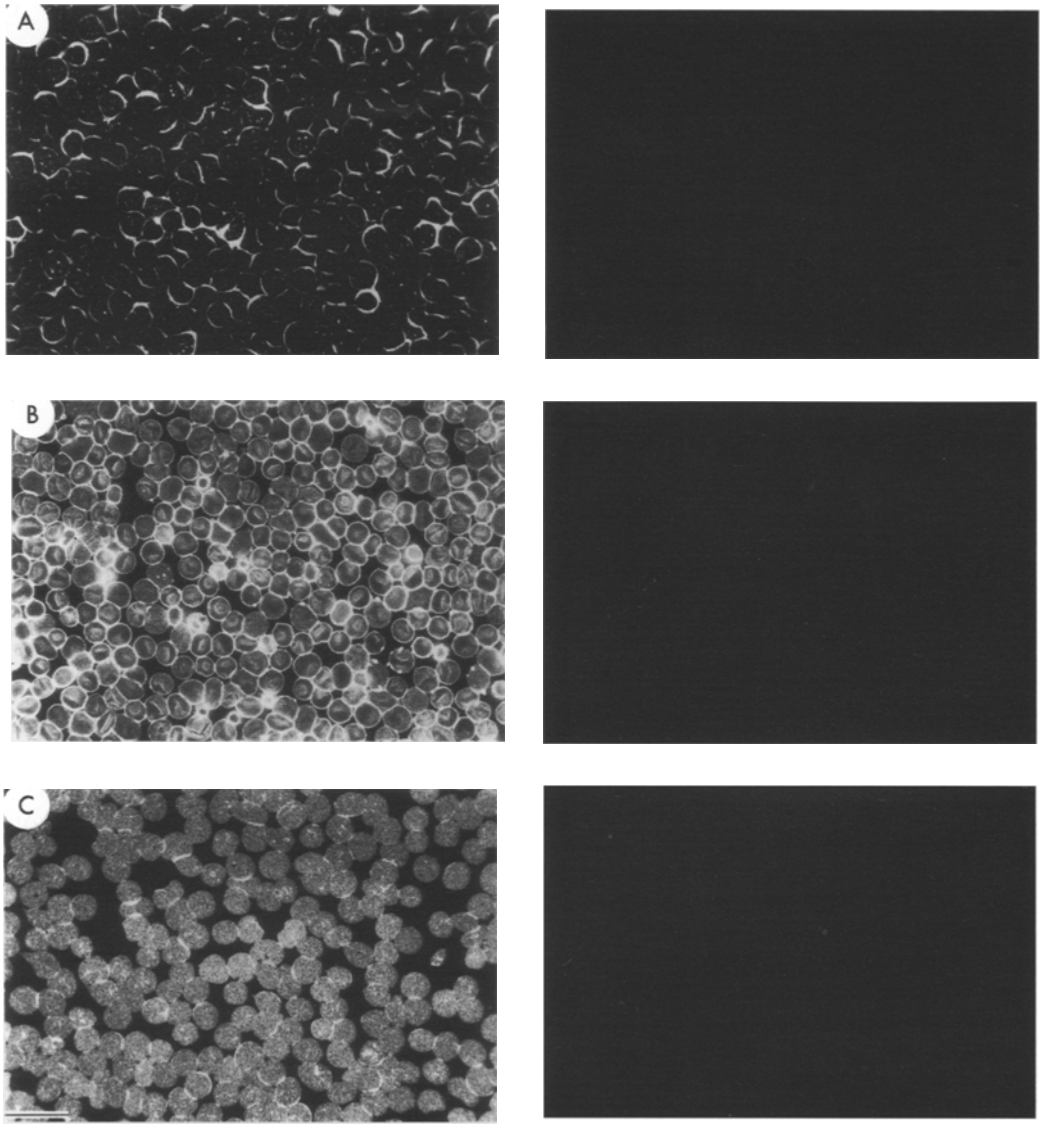

Fig. 6. Membrane fragments in RBC-PM lawns are oriented with their cytoplasmic face available to the medium. RBC-lawns and RBC-ghost lawns were fixed with 1% glutaraldehyde and processed for indirect immunofluorescence using antibodies directed to determinants located on the extracellular (blood group A antigen) or the cytoplasmic (spectrin) surfaces of the membrane. (*A*) Immunofluorescence images of RBC-PM lawns treated with anti-blood group A (left) and control (right) monoclonal antibodies. (*B*) Immunofluorescence images of RBC-ghost lawns treated with anti-blood group A (left) and control (right) monoclonal antibodies. (*C*) Immunofluorescence images of RBC-PM lawns treated with polyclonal anti-spectrin IgG (left) and a nonimmune control (right). Bar is 20 μm in length

The ability of HRBC-PM to bind CVs was assessed as a function of CV concentration and compared to that of egg PM lawns, as described in Materials and Methods. The results of this experiment (Fig. 7) were unambiguous: CVs were unable to bind to the RBC-PM lawns. This result is consistent with the notion that binding of CVs to egg PM lawns requires the presence of proteins that are not found on the RBC-PM.

We have also observed that *S. purpuratus* CVs can bind to PM lawns prepared from *S. droebachiensis*, *A. punctulata*, and *L. pictus* eggs. Quantitation revealed that *S. purpuratus* CVs bound better to *S. purpuratus* PM lawns than to *L. pictus* PM lawns (*data not shown*); however, at present it is not clear whether this difference is significant, or simply due to the fact that binding varies somewhat from one preparation to the next (*see* Fig. 1).

Specificity of CV Binding: Binding of Heterologous Vesicles

An additional way of investigating the specificity of CV binding is to alter the source of the binding vesicles. If CV binding to PM lawns is a specific protein-mediated event, liposomes and cellular organ-

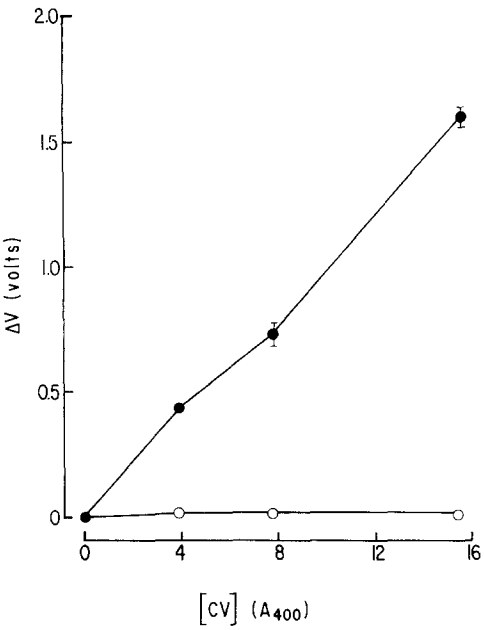

Fig. 7. CVs do not bind to red blood cell (RBC) PM lawns. RBC-PM lawns were prepared from washed human RBCs as described in Materials and Methods. CV binding to egg-PM lawns (filled symbols) and RBC-PM lawns (open symbols) was assayed as described in Materials and Methods. Results are the mean ± SD of triplicate samples, and are representative of three similar experiments

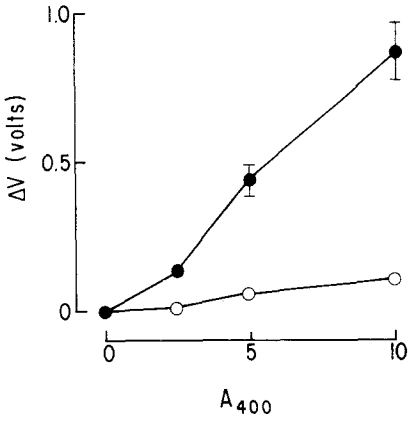

Fig. 8. Yolk vesicles do not bind to PM lawns. A yolk platelet fraction, prepared by differential centrifugation of an egg homogenate, was resuspended in CV preparation buffer at the indicated concentration, and tested for binding to *S. purpuratus* PM lawns (open symbols). For comparison, CV binding was also tested (filled symbols). Results are the mean ± SD of triplicate samples, and are representative of three similar experiments

elles that do not associate with the PM in vivo should not bind to egg PM lawns.

In this regard, yolk platelets, which are the most abundant organelle of the egg and are of approximately the same size as CVs, were of particular interest. Yolk platelets were prepared by a modification of the procedure described by Ii et al. (1978). This procedure, which yields a crude yolk platelet preparation that contains some CVs, was chosen because we wanted a procedure that was quick (to decrease the possibility of surface proteolysis), and compatible with our methods for preparing CVs and PM lawns. The results presented in Fig. 8 show that some vesicles from the yolk platelet preparations did bind to egg PM lawns. The average level of binding in this and two additional experiments was low (13.8 ± 0.3% of CV binding) but sufficient to warrant further investigation. Therefore, PM lawns which had been incubated with a yolk platelet preparation as described in the legend to Fig. 8 were fixed, permeabilized with Triton X-100, and subjected to immunofluorescence analysis with a monoclonal antibody to hyalin (a CV content protein). Comparison of phase contrast and fluorescence photos of these preparations demonstrated that 74 ± 9% of the bound vesicles were CVs (n = 2 preparations with at least 500 vesicles counted in each preparation). Thus, the small number of CVs that contaminate the yolk platelet preparation are responsible for most of the binding observed in Fig. 8. Based on these data, it is clear that yolk platelets bind very poorly to egg PM lawns. In other experiments, we have observed that dioleoylphosphatidyl choline liposomes also are unable to bind to egg PM lawns (*data not shown*).

Discussion

Previously we have shown that cortical secretory vesicles purified from the eggs of *S. purpuratus* can bind to fragments of egg PM to form a reconstituted cortex preparation known as a reconstituted lawn or RL (Crabb & Jackson, 1985). When bathed in Ca^{2+}-containing buffers, RLs undergo a morphological change which appears by several criteria to be equivalent to the in vitro exocytosis reaction in cortical lawn preparations of egg cortex (Crabb & Jackson, 1985). The ability to reconstitute exocytosis from isolated CVs and PM permits these two components to be independently manipulated, and thus should permit investigation of the molecules responsible for CV binding and exocytosis. The quantitative binding assay described above will be an important tool in these investigations.

Although the binding assay is based on the same principle (the ability of CVs to scatter light in the dark field microscope) as the exocytosis assay described by Zimmerberg et al. (1985), the two assays use different biological preparations and quantitate fundamentally different parameters. The exocytosis assay utilizes a cortical lawn preparation with an intact compliment of cortical vesicles and measures exocytosis, whereas the binding assay

utilizes purified PM and CV preparations, and quantitates CV binding. Furthermore, unlike exocytosis, analysis of CV binding requires comparison of data obtained from one sample with that obtained from others. For this reason it was necessary to precisely standardize conditions. This included: (*i*) preparing PM lawns that are confluently coated with egg PM fragments, and defining standard conditions for reassociation and washing. (*ii*) demonstrating that within the spot from which data was recorded, the signal is proportional to the surface area, and (*iii*) calibrating the light scattering apparatus with a series of scattering standards. The binding assay is very sensitive, in the sense that CV binding is measured from a spot only 354 μm in diameter. It is also quite tedious, since individual RLs must be analyzed one at a time with the photodiode apparatus. Using the binding assay we have obtained evidence which suggests that the reassociation of CVs with egg PM is a specific protein-mediated event.

Proteolytic digestion of PM fragments (Fig. 3) inhibited binding. This observation strongly suggests that reassociation requires proteinaceous components located on the cytoplasmic surfaces of the PM. Based on the large amount of actin in cortex (Begg & Rebhun, 1979; Spudich & Spudich, 1979; Mabuchi, Hosoya & Sakai, 1980; Otto, Kane & Bryan, 1980; Vacquier & Moy, 1980) and PM lawn preparations (Kopf et al., 1982; Vater & Jackson, 1989*b*), it is tempting to suggest that actin may be involved in tethering CVs to the PM. Filamentous actin is present in microvilli, but also forms a network along the inner surface of the PM of the unfertilized egg (Chandler, 1984; Sardet, 1984; Henson & Begg, 1988; Spudich, Wrenn & Wessells, 1988; Bonder et al., 1989). Spectrin is also enriched in the cortical region of the egg (Schatten et al., 1986; Bonder et al., 1989), and could couple CVs to the actin network. However, it has also been shown that cytoskeletal toxins such as cytocholasin B have no effect on exocytosis in CLs (Whitaker & Baker, 1983), and do not dislodge CVs from the plasma membrane, either in intact eggs (Vacquier & Moy, 1980) or cortical lawn preparations (Whitaker & Baker, 1983). Furthermore, it has been observed (without quantitation) that DNase I, cytochalasin B, phalloidin and cholchicine did not block CV binding to egg PM lawns (Crabb, 1986).

With regard to the specificity of binding in the in vitro system, we have shown that CVs bind to fragments of egg PM under conditions in which heterologous vesicles (yolk platelets and liposomes) will not. Conversely, we have shown that CVs cannot bind to PM fragments prepared from HRBCs, but will bind to PM fragments prepared from the eggs of other species of sea urchin. These results are consistent with the notion that binding in the in vitro system may be a specific event. They are supported by previous data which show (*i*) that isolated CVs are unresponsive even to high concentrations of Ca^{2+} unless they have been bound to PM (Crabb & Jackson, 1985; Whalley & Whitaker, 1988); and (*ii*) that the Ca^{2+}-induced morphological change in both CLs and RLs is accompanied by the vectorial transport of CV contents across the egg PM (Crabb & Jackson, 1985).

On the other hand, they fall short of proving that in vitro reassociation correctly reconstitutes the CV-PM junction. The fact that the threshold Ca^{2+} concentration required for half-maximal reaction is higher in RLs than in CLs suggests that RLs are somewhat impaired, presumably due to the potentially detrimental conditions used in preparing the CV and PM fractions. Based on calculated Ca^{2+} concentrations, we originally reported Ca^{2+} thresholds of 18 and 6 μM, respectively, for RLs and control CLs (Crabb & Jackson, 1985). In more recent experiments, in which we utilized a Ca^{2+} electrode and redesigned perfusion chambers, we obtained a Ca^{2+} threshold of 36 μM for RLs, and 6 μM for CLs. Whalley and Whitaker (1988) have reported that *L. pictus* RLs also have a higher Ca^{2+} threshold than CLs. They report Ca^{2+} thresholds of 25 and 6.3 μM for *L. pictus* RLs and CLs, respectively. While Whalley and Whitaker's (1988) threshold value for *L. pictus* RLs compares favorably with our figure of 36 μM for *S. purpuratus* RLs, it should be noted that they utilized a lower ionic strength, lower pH buffer to prepare CVs. CVs prepared with a higher ionic strength, higher pH buffer produced *L. pictus* RLs with higher thresholds (250 μM Ca^{2+}).

In summary, the binding assay described in this report should prove useful in elucidating the molecular basis for cortical exocytosis. The results of protease inhibition and binding specificity experiments are consistent with the hypothesis that binding of CVs to egg PM lawns is a specific protein-mediated event. Along with previously presented data (Crabb & Jackson, 1985), they support the authenticity of the reconstitution process.

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