Exocytosis of Sea Urchin Egg Cortical Vesicles In Vitro Is Retarded by Hyperosmotic Sucrose: Kinetics of Fusion Monitored by Quantitative Light-Scattering Microscopy

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ABSTRACT We have used the isolated planar cortex of sea urchin eggs to examine the role of osmotic forces in exocytosis by morphological and physiological methods. Electron micrographs of rotary-shadowed replicas show an *en face* view of exocytosis and demonstrate fusion of cortical vesicles to the underlying oolemma upon addition of calcium. Freeze-fracture replicas of rapidly frozen cortices reveal specialized attachment sites between cortical vesicles and the oolemma, and between the cortical vesicles themselves. We describe a novel light scattering assay for the kinetics of fusion which allows rapid changes of solutions and monitors exocytosis in real time. The rate and extent of fusion are found to be calcium dependent. The removal of calcium halts exocytosis.

The validation of exocytosis in this system and development of tools for kinetic analysis allowed us to test two predictions of the osmotic hypothesis of exocytosis: hyperosmotic media should inhibit exocytosis; calcium should cause vesicular swelling. Cortical vesicles were found to be permeant to sucrose, glucose, and urea. In media made hyperosmotic with 1.7 M sucrose, cortical vesicles were seen to shrink. Addition of calcium in hyperosmotic media led to a 10-fold decrease in the rate of exocytosis compared with the isotonic rate. The rate, while triggered by calcium, was no longer calcium-dependent. This slowing of exocytosis allowed us to photograph the swelling of cortical vesicles caused by calcium. Removal of calcium had no effect on subsequent exocytosis. Return of cortices to isotonic medium without calcium led to immediate exocytosis. These results are consistent with the idea that swelling of cortical vesicles is required for fusion of biological membranes.

Exocytosis, cell–cell fusion, and enveloped virus infection share in common the fusion of two bilayer membranes. Investigation of a model system—fusion of phospholipid vesicles to planar phospholipid membranes—reveals two important steps in fusion (8, 10, 44, 45). (*a*) Membranes must achieve intimate contact in a prefusion state, and (*b*) the vesicles must swell in order to fuse (2, 9). This requirement suggests that osmotic vesicular swelling drives fusion by exposing hydrophobic domains in attached bilayers, allowing their intermixing and fusion (2). In this paper we ask if the mechanical stress provided by osmotic swelling, which works to stretch the membrane, is also needed in biological exocytosis.

There have been many observations consistent with a requirement for vesicular swelling in exocytosis. Increasing the osmolality of media bathing sea urchin eggs, chromaffin cells, parathyroid cells, or platelets inhibits stimulus-induced exocytosis (5, 12, 15, 28, 46). The problem with these observations, however, is that hyperosmotic treatment of cells leads to shrinkage not only of the secretory vesicles but also the entire cell, with associated changes in the concentrations of all impermeant species, possible disruption of cytoskeletal–membrane relationships, and unpredictable increases in cytoplasmic viscosity. Osmotic gradients do inhibit antidiuretic hormone–induced fusion without altering cell volume (18) and inhibit permeabilized chromaffin cells (20). However, the osmotic hypothesis of exocytosis can be better tested in a cell-free system where one can easily control the electrolyte and nonelectrolyte concentrations, viscosity, and osmolality of the solutions bathing the interacting membranes.

The planar isolated cortex of the sea urchin egg meets those conditions and is a favorable preparation to test this hypothesis. In the intact egg, intracellular calcium rises shortly after fertilization and is followed by a massive exocytosis of approximately ten thousand cortical vesicles per egg (35, 42). The planar isolated cortex of this egg can be prepared by shearing the eggs stuck to poly-L-lysine–coated surfaces with a jet of an appropriate medium. Since the force that adheres eggs is greater than the force that maintains cell integrity, the eggs lyse, leaving attached to the surface large planar areas of the plasma membrane–cortical granule complex which can then be exposed to the experimental medium (31, 40). Subsequent elevation of the free calcium level in the medium leads to cortical vesicle disappearance (49), which in early scanning electron micrographs was suggestive of vesicle–vesicle fusion (41). In improved isolation media, more akin to the cytoplasmic milieu, micromolar free calcium leads to exocytosis (3, 43).

If osmotic, swelling is the driving force for initiation of biological membrane fusion, then two predictions can be tested: (*a*) hyperosmotic treatment of exocytotic systems should inhibit exocytosis, and (*b*) compounds that trigger exocytosis, such as calcium, should induce vesicle swelling. Preliminary experiments to test the effect of hyperosmotic treatment on exocytosis of these cortices indicated that hyperosmotic pretreatment with sucrose dramatically reduced the rate of exocytosis. To better study this inhibition, we first examined the ultrastructure of the isolated cortical layer, the relationship between cortical vesicles and the plasma membrane, and demonstrated fusion of the cortical vesicles with the underlying plasma membrane in vitro. We then designed a chamber for rapid changes of viscous solutions, and developed a light-scattering method to monitor exocytosis quantitatively and continuously in real time to study the effect of hyperosmotic sucrose solutions on the exocytotic event. In this report we describe these effects and also document the swelling of cortical vesicles induced by calcium.

MATERIALS AND METHODS

Making Cortices: *Strongylocentrotus purpuratus* adults were obtained from the Olympic Peninsula, Washington, and Point Arena, California, and *Lytechinus pictus* adults were obtained from Pacific Biomarine, Venice, CA. The specimens were maintained in running seawater tanks at the Hopkins Marine Station. Unless mentioned, all experiments were performed with *S. purpuratus*.

Eggs, obtained by intracoelomic injection of 0.5 M KCl, were spawned into fresh seawater. They were passed once through an 85-μm nitex mesh, washed three times, and then stirred in fresh seawater with a 60 rpm clock motor in a 0.5% suspension at 12°C. Sperm was collected as the concentrated semen and stored at 0–4°C in a closed plastic tube. Fertilizability was checked by addition of 1 ml of 1:5,000 sperm in seawater to 1 ml of egg suspension. Only egg preparations yielding >99% fertilization were used.

Cortices were prepared for kinetic assay essentially according to the method of Vacquier (40). A drop of a fresh 1 mg/ml poly-L-lysine solution was placed on a coverslip. After 2 min, coverslips were washed extensively in distilled water and allowed to dry. After the eggs were dejellied by bringing the pH to 5.3 for 2 min, the eggs were rinsed in a large volume of millipore-filtered (0.45 μm) seawater, hand centrifuged, resuspended to a 30% suspension, and deposited on a coated coverslip (coverslip kept on ice). After 1–10 min excess eggs were removed by gentle rinsing with ice cold calcium-free artificial seawater (454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 27.1 mM $MgSO_4$, 4.4 mM $NaHCO_3$, 10 mM HEPES, pH 8.0). The remaining attached eggs were then subjected to the shear force of an ice-cold stream of a solution simulating the intracellular milieu consisting of 250 mM potassium gluconate, 250 mM *N*-methyl-D-glucamine, 180 mM acetic acid, 50 mM PIPES, 5 mM NaCl, 1 mM free Mg^{++} as $MgCl_2$, and 5 mM EDTA, pH 6.7, hereafter referred to as cortex medium (38), delivered through a 23-gauge hypodermic needle with a 10-cc syringe. Just enough pressure was applied to the syringe to lyse the eggs. Coverslips containing the cortices were quickly transferred to a perfusion chamber (Fig. 1) and viewed under dark field (light-scattering) microscopy with a Zeiss phase contrast microscope (Carl Zeiss, Inc., Thornwood, NY). Isolated cortices were washed with 5 ml of cortex medium after the chamber was sealed. Good dark field was effected with the phase contrast condensor designed for the 100× objective used in conjunction with a 10× objective. Although the scattering angle was not determined, vesicles appeared as bright spots against a black background (Fig. 5*c*). Thus light intensity was never a problem.

Measuring Exocytosis: After focusing, we directed the scattered light to a photovoltaic cell (PV-100, EG&G, Salem, MA) positioned in the camera tube above a 10× ocular to collect all of the light. The signal from the photocell was amplified with an operational amplifier (AD-48J, Analog Devices, Inc., Norwood, MA) and displayed on a chart recorder. Temperature was also recorded continuously on the recorder, as detected by a thermistor in the perfusion chamber. We adjusted incident light intensity to yield full scale pen deflection on the chart recorder (Fig. 1). We then added test solutions sequentially by sucking them through the perfusion chamber with negative pressure, supplied by a syringe. The resultant changes in scattered light were recorded. After the reaction had reached an end-point, as judged by no further changes in scattered light for 30 s, water was perfused through. This burst the remaining vesicles and provided a zero point for normalization of data. Thus the contribution to the signal by organelles other than cortical vesicles was insignificant. All measurements were performed in a 16°C room (Forma Scientific, Marietta, OH).

Cortices were also monitored directly using an 100× oil immersion objective, 10× ocular, and video-tape recording or 35-mm photomicrography (Kodak tech-pan and tri-X) (Fig. 1). Cortical vesicle fusion with the oolemma and cortical vesicle lysis could be readily distinguished in differential interference microscopy by the presence of domes (see Results). Playback of the video-tape with single frame display allowed counting of the number of vesicles in a selected area as a function of time. Areas of the cortical vesicles were also estimated directly from the 35-mm negatives of a single area of cortex as a function of time. Negatives were exposed, developed, and handled identically. A portion of each sharply focused negative was scanned by a micro densitometer. The resultant 0.1-μm resolution digitized image was stored and analyzed with a Vax 11/780 with a DeAnza IP8500 imagery system. Software developed at the National Institutes of Health was used to measure the area of each vesicle up to the threshold using a simple fill algorithm (21). To avoid bias, the samples were randomly presented to an impartial observer who selected the threshold density for the granule edge. Since the cortices didn't move, we were able to measure the area of many identical cortical vesicles throughout the experiment. These were tested for statistical differences with a Kolmogorov-Smirnov two sample test (29).

Free calcium and magnesium levels were maintained with EDTA, $CaCl_2$, and $MgCl_2$ (23). Calcium activity was determined directly with an ion-selective electrode (30), with a series of published calibration solutions (39). Measured activities were read as concentrations of free calcium in 0.1 M KCl.

We measured β-glucanase release from the control vesicles by the technique of Moy et al. (25). Cortices were also pretreated with 20 μM A23187 in 500 mM glycine, 250 mM potassium glutamute, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM EGTA, and 10 mM HEPES, pH 6.7 to obviate the contribution of sequestered calcium in a suspected wave of exocytosis (3).

Hyperosmotic media were prepared by weighing the appropriate amount of sucrose and making the solutions up to volume with the isotonic cortex media containing the desired calcium activities. Each solution was titrated to the calcium activity of the corresponding isotonic cortex medium with 0.5 M EDTA 0.2 M $MgCl_2$. This yielded media of identical calcium activity regardless of osmotic strength. Osmotic pressure was taken directly from the Handbook of Chemistry and Physics, 59th edition except for the highest osmotic condition (2.0 M) which was extrapolated from that handbook. Viscosity was measured with a Hoeppler precision viscometer of falling ball design. Inorganic chemicals were of reagent grade (J. T. Baker Chemical Co., Phillipsburg, NJ); organic chemicals were from Sigma Chemical Co. (St. Louis, MO). Calcium for electrode calibration was added from a prepared solution of 0.1 M $CaCl_2$ (Orion Research Inc., Cambridge, MA).

Electron Microscopy: For thin section electron microscopy and replication of whole cortices after critical point drying, cortices were made by shearing eggs attached on plastic (for sections) or glass (for replica) coverslips as described above but at room temperature. They were fixed for 1 min in 1% glutaraldehyde (Polysciences, Inc., Warrington, PA), 1% paraformaldehyde, 400 mM NaCl, 50 mM potassium phosphate buffer (pH 7) with 50 mM lysine added just before use (4). They were then transferred and fixed for 1 h in the same fixative without lysine, and then postfixed for 1 h in 1% OsO_4 in 400 mM NaCl, 50 mM potassium phosphate buffer (pH 6).

To prepare thin sections, cortices were dehydrated in acetone, embedded in Spurr's resin, and sectioned *en face* (27). For critical point drying, cortices were dehydrated in absolute ethanol and bone-dry liquid CO_2; they were then replicated by rotary shadowing with platinum and reinforced with carbon essentially as described (1).

For freeze fracture, cortices made on small bits of glass were immediately fast frozen by slamming them onto a helium-cooled copper block (16). The preparations were subsequently fractured (−150°C), shadowed with platinum, reinforced with carbon, and examined.

RESULTS

Structure of the Unfertilized Egg Cortex

In phase-contrast microscopy, the planar cortex appears as a monolayer of cortical granules (Fig. 5*A*). Electron micrographs of cortices (prepared by shearing stuck eggs with isoosmotic solutions containing 0.1 μM calcium) reveal a membranous reticulum and closely packed cortical granules, often in contact with each other (Fig. 2*a*). We examined the relationship between the cortical granules and the plasma membrane after fast freezing, fracturing, and replicating unfixed cortices within a minute of their isolation (Fig. 2, *b*, *c*, and *d*). Fracture planes through the plasma membrane reveal the presence of specialized zones of contact between the cortical granule and plasma membrane. These areas are free of intramembranous particles in the area of contact between cortical granules and plasma membrane (Fig. 2, *b* and *c*). Specialized contact sites are also observed between some cortical granules themselves (Fig. 2*d*).

Ultrastructural Evidence for Exocytosis

Rotary shadowing is particularly useful in distinguishing topologically separate surfaces from continuous ones, because platinum shadow accumulates on surfaces (in this case the surface membrane of the vesicles and the plasma membrane.) Thus cortical vesicles lying on top of the plasma membrane

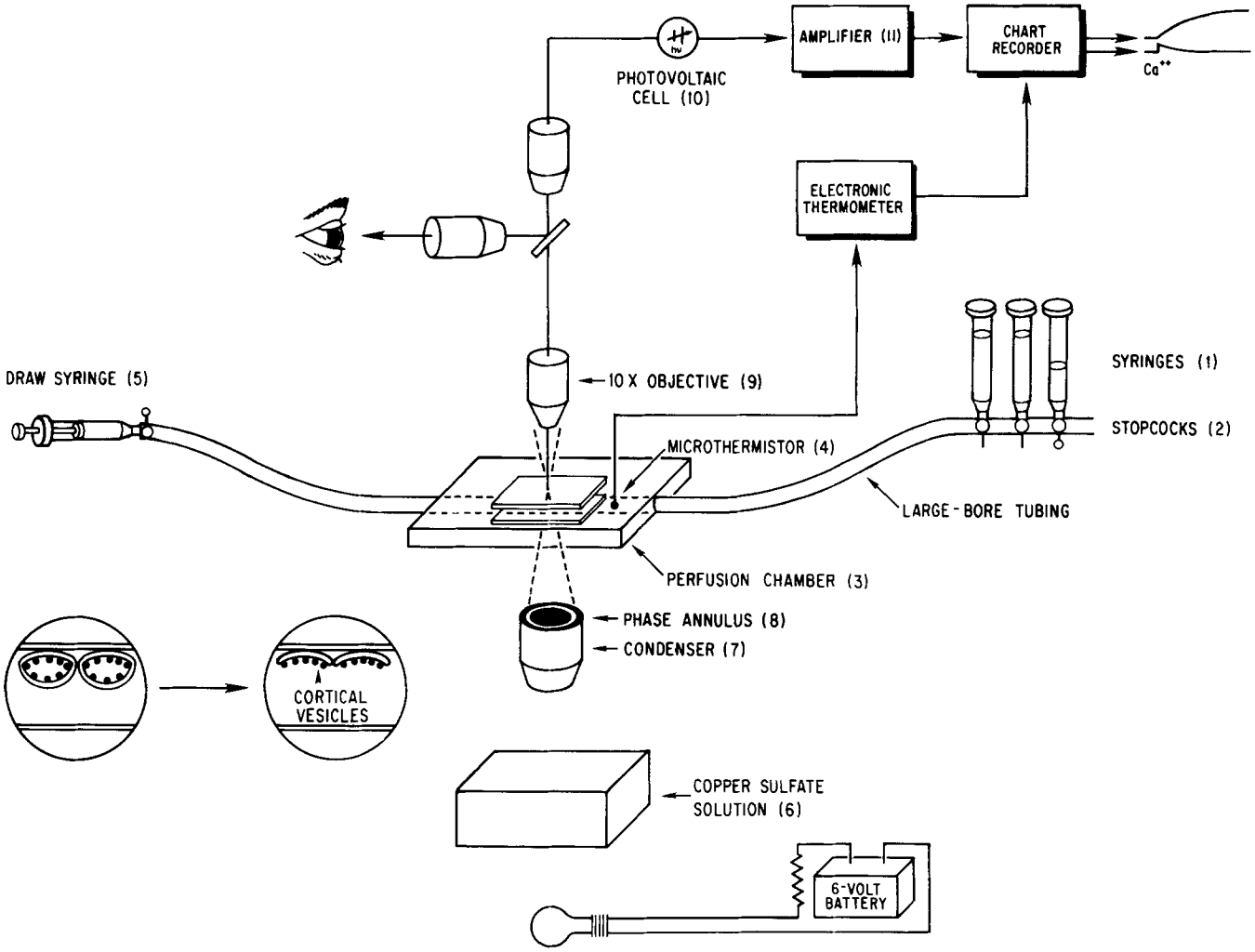

FIGURE 1 Schematic drawing of the apparatus. Solutions are placed in syringes (*1*), selected with stopcocks (*2*), and flowed through large bore tubing to the perfusion chamber (*3*), which is fitted with a microthermistor (*4*). Glass coverslips line the top and bottom of the chamber, and cortices are prepared on the upper coverslip, facing down. A change of solutions is initiated by pulling on a syringe (*5*). A stopcock facilitates syringe emptying. Light from an halogen bulb powered by a 6-V lead storage battery passes through a copper sulfate solution (*6*), a condenser (*7*), the phase annulus for a 100× oil immersion objective (*8*), and the preparation. Some of the scattered light is collected by a 10× objective (*9*) and alternatively visualized through oculars or passed through the camera tube. The tube is fitted with a PV-100 photovoltaic cell (*10*). The light signal is amplified (*11*) and recorded on a two-channel chart recorder along with chamber temperature. The slight temperature transient denotes the time of solution change. Alternately, a bubble placed between solutions heralds entry of the new medium. Very short pulses of solution can be accurately made by filling varying lengths of tubing with the test medium and flanking them with the control medium.

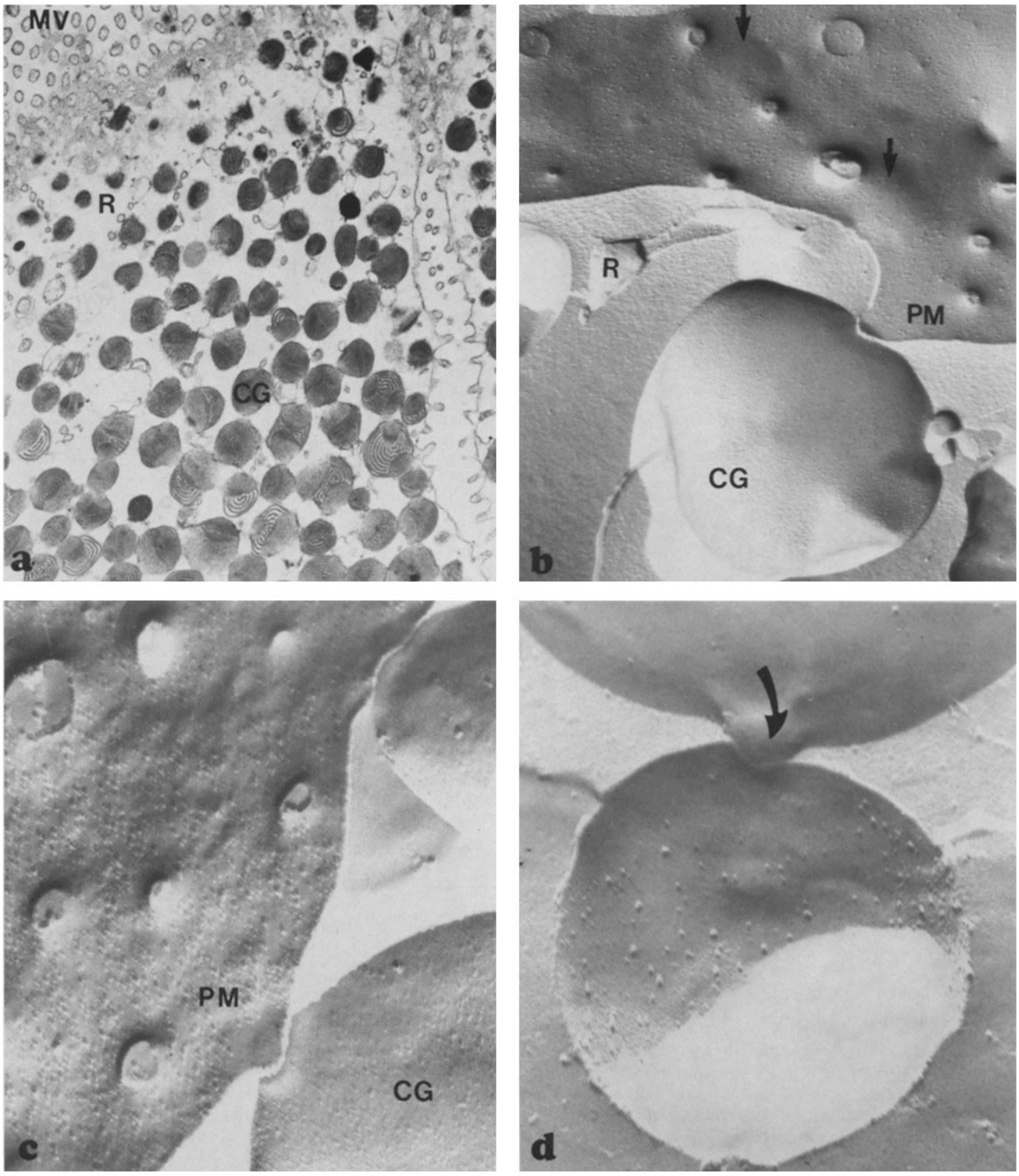

FIGURE 2 Unfertilized sea urchin egg cortex. (*a*) Thin section of two stuck down cortices. The slightly oblique tangential section passes through a zone of microvilli (*MV*), a zone where a membrane reticulum (*R*) is abundant, and a zone where cortical granules (*CG*) are in close opposition. Thin section. × 8,100. (*b*) Fast frozen, fractured replica of a cortex showing the area of contact between the P face of plasma membrane (*PM*) and a cortical granule (*CG*). Arrows show two areas poor in intramembranous particles under which cortical granules are probably located. Elements of the membranous reticulum are also seen (*R*). Fast freeze fracture. × 44,200. (*c*) Detail of the contact site between plasma membrane (*PM*) and cortical granule (*CG*). Fast freeze fracture. × 92,000. (*d*) Contact site (arrow) between two cortical granules. Fast freeze fracture. × 112,000.

appear with a characteristic dark ring of shadow (Fig. 3*d*). Exposure of the isolated cortices to 17 μM Ca^{++} in the perfusing milieu results in the disappearance of most of the individual cortical vesicles and the appearance of domes which lift the membraneous layer away from the poly-L-lysine-coated support. These domes are clearly continuous with the plasma membrane. To see individual fusion events, cortices were sheared forcibly, fixed at 5–10 s after exposure

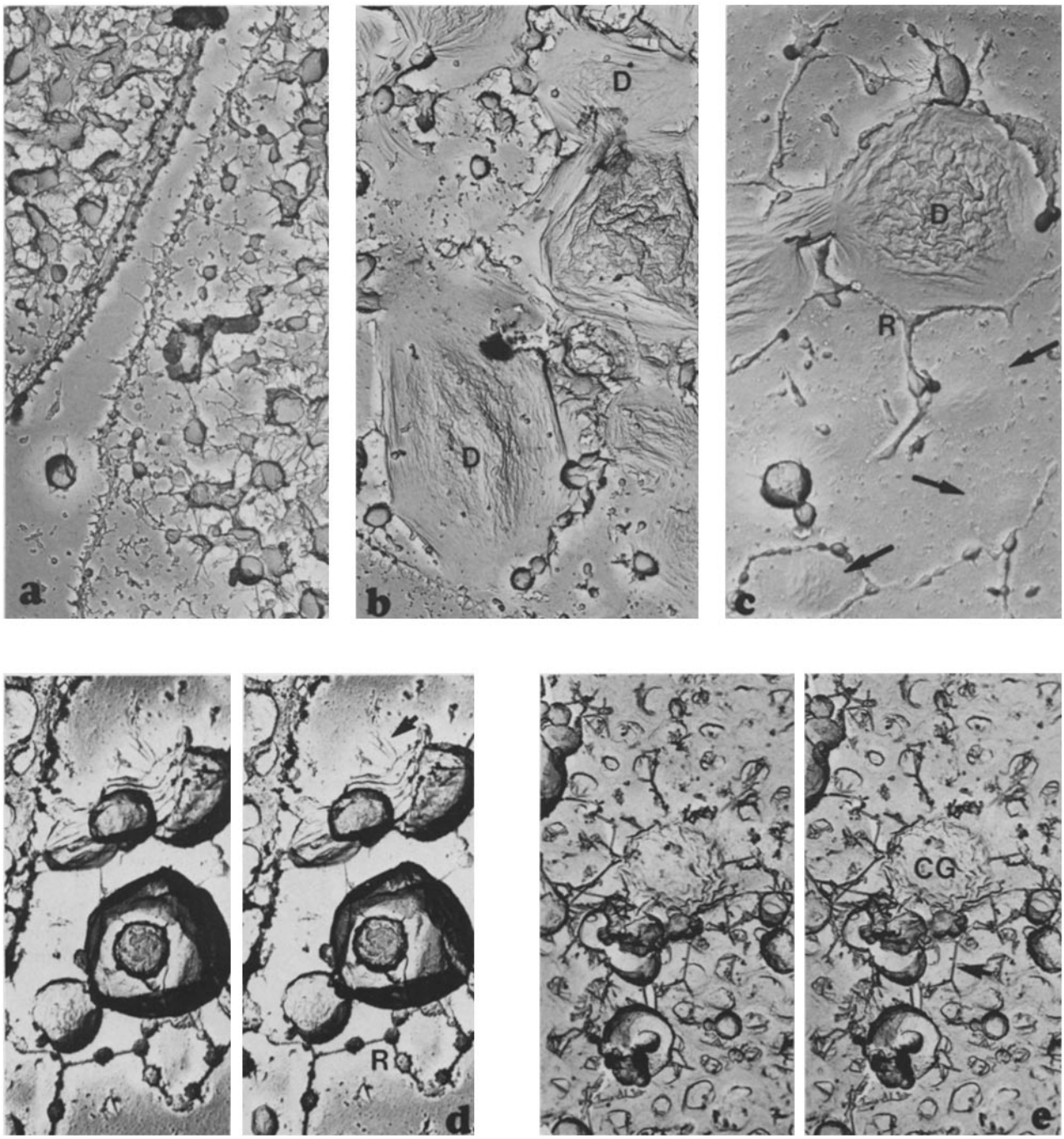

FIGURE 3 Evidence for vectorial fusion of cortical granule and plasma membranes: replica of fixed, critical point dried, rotary shadowed preparations of isolated planar cortices. For clarity, cortices were sheared forcibly to detach many granules. (*a*) Edges of two unfertilized cortices in intracellular milieu containing 0.1 μM Ca^{++}. Cortical granules, elements of a vesicular reticulum, and microfilaments are seen. Note the edge of the cortex at right has lost many granules, probably detached upon shearing. × 5,300. (*b*) A cortex that has been exposed 30 s to a perfusion medium containing 17 μM Ca^{++}. Domes (*D*) of various sizes have lifted up part of the attached cortex and granules have disappeared. The folds and flattening in domes may be artifacts due to fixation and drying of the preparation. × 4,400. (*c*) A cortex exposed to 17 μM Ca^{++} for 30 s. Besides small domes (*D*) and elements of the membranous reticulum (*R*), fusion sites of single cortical granules are clearly seen (arrows) in the midst of the stuck down plasma membrane (little dots and holes are microvilli seen face on). × 9,000. (*d*) Stereo pair. Edge of an unfertilized egg cortex showing the membranous reticulum (*R*) and granules. Arrow shows microfilaments. × 12,000. (*e*) Stereo pair. A small cortical granule (*CG*) fusing with the plasma membrane. Note the inside of microvilli and the profusion of short actin filaments (arrow). × 15,000.

to Ca^{++}, critical point dried, and then rotary shadowed. They show small domes in the size range of single cortical granules (Fig. 3*e*). Samples fixed at 30 s after calcium addition show much larger domes (Fig. 3, *b* and *c*). The smaller domes have also flattened out. At later times (>1 min), the domes tear and open (data not shown).

We feel the following sequence of events would lead to the observed morphological changes. First, the granules would

fuse with the underlying plasma membrane (Fig. 3, *c* and *e*). The granule content would continue to swell lifting up the underlying continuous membrane layer. Domes would form and eventually coalesce (Fig. 3, *b* and *c*).

These domes are easily seen in differential interference microscopy by focusing through the domes. Domes appear ballooned out, indicating that the dehydration of the cortex before critical point drying results in the crinkled appearance of the domes in Fig. 3. We use the appearance of domes as a criterion for exocytosis in the subsequent experiments. Vesicle bursting does not result in domes.

Different Assays for Exocytosis

The exocytotic reaction in the isolated cortices has been previously measured by three different methods: (*a*) turbidity changes of suspended cortices prepared by homogenization (14, 33), (*b*) direct visualization of the disappearance of cortical vesicles in planar cortices by phase contrast microscopy (37, 43), or (*c*) measuring release of β-glucanase, a cortical vesicle enzyme (25). We tried to assess exocytosis in hyperosmotic media with these different techniques. The effects of hypertonic media on exocytosis could not be studied with the suspended cortex method since the cortices floated on the dense sucrose medium, and accurate turbidimetry was impossible. Measurement of β-glucanase appearance in the bathing medium (Fig. 4) reflected the true rate of exocytosis for low calcium levels and slow rates, as previously reported (25). However, with higher calcium activity, glucanase release lagged far behind exocytosis (as ascertained by comparing direct visualization of cortical exocytosis with β-glucanase release). As noted (25), diffusion of the enzyme from under the surface of the cortices is most likely the reason for the delay. This difference was accentuated in hyperosmotic milieu and unaffected by pretreatment with the calcium ionophore A23187 (20 μM) (Fig. 4). Exocytosis could be monitored with minimal perturbation by video light microscopy, and the kinetics of the process could be quantified with single-frame video playback. Phase-contrast high magnification microscopy gave a distinct image of each vesicle (Fig. 5*A*), and upon addition of calcium, vesicles disappeared (Fig. 5*B*). In video single frame playback the sharply bordered image of each vesicle disappeared in an explosive fashion, leaving behind the apparently unbounded vesicle contents which then dispersed radially. With this procedure, therefore, exocytosis is seen as phase-dense vesicle disappearance. We determined the number of vesicles at various times in the reaction with video recording and single frame playback. Although unambiguous, intense light levels were needed, counting of vesicles was time consuming and tedious, and only one cortex (~5,000 vesicles) could be viewed at a time.

Our approach for measuring kinetics (Fig. 1) is based on the finding that under dark-field low magnification microscopy each cortical vesicle becomes a scattering center against a dark background (Fig. 5*C*), whereas the dispersed vesicle contents have little light scattering potential (Fig. 5*D*). The scattering can be quantitatively measured by a photovoltaic cell positioned in the camera tube of the microscope, yielding a linear voltage output proportional to the scattered light plus background. This voltage, recorded as a function of time, provides a continuous record of the number of granules at any time (such as after addition of Ca^{++}, Fig. 6). Under the described conditions, ~10^6 secretory vesicles (100 cortices)

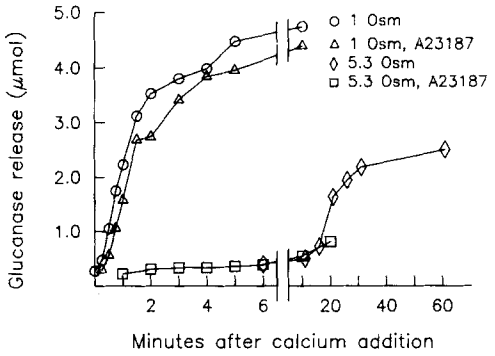

FIGURE 4 Dejellied eggs were allowed to settle on four 60 × 15-mm protamine-coated petri dishes on ice. After 2 min they were gently rinsed with ice-cold calcium-free seawater and then sheared with a jet of ice-cold buffer (250 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 10 mM EDTA, 10 mM HEPES, 10 mM $MgCl_2$, pH 6.7). After extensive washing with buffer, two of the dishes were incubated for 5 min in buffer made 20 μM in calcium ionophore A23187. Two dishes were pretreated with 2 M sucrose buffer. Finally all dishes were drained of media and treated with 17 μM Ca^{++} in either buffer (1 Osm) or 2 M sucrose-buffer (5.3 Osm), warmed to 19°C and swirled continuously. At various times 0.2-ml samples were withdrawn. Glucanase was estimated in these samples by measuring the release of glucose from laminarin in an overnight incubation. Glucose was measured with a glucose oxidase assay. Total released glucanase was summed over previous samples.

fusing to plasma membrane can be monitored per experiment. Another advantage of this system is that low light levels can be used, which minimizes specimen warming; also, the large depth of field associated with low magnification alleviates the concern that granules are merely moving out of focus rather than under-going exocytosis. To rapidly change solutions, a perfusion chamber with large diameter tubing was used (Fig. 1), which allowed rapid (<17 ms) changes of solution. Distilled water, added at the end of the reaction to lyse the remaining granules, was used to calibrate for both background and for normalization of data. The kinetics obtained by low power light-scattering microscopy compared well with actual counts of granules on video records under similar conditions (Fig. 9*A*).

Kinetic Dependence of Exocytosis upon Calcium

When the isolated planar cortices were perfused with cortex media containing more than 0.5 μM calcium, exocytosis ensued. Fig. 6 shows a typical measurement. In this experiment, initiated by perfusion with 17 μM Ca^{++}, a change in scattering begins immediately upon addition of Ca^{++} and is one-half completed at ~14 s. At the end of the experiment, we burst remaining vesicles by perfusion of the preparation with distilled water. As seen, light-scattering decreased by an additional one-third of the total change. Thus, in this experiment, 17 μM Ca^{++} induced 66% of the cortical vesicles to fuse. Fig. 7*A* shows the percentage exocytosis from many such records, when calcium was added 2 min after shearing.

The rate of exocytosis was clearly Ca^{++} dependent. Fig. 7*B* shows the rate expressed as $1/t_{1/2}$ versus calcium concentration. As seen, the data can fit two straight lines, suggesting that the reaction of calcium and cortices is pseudo-first-order at high concentrations and at physiological concentrations, but involve two different affinity sites.

The rate and extent of exocytosis diminished after shearing in cortex medium. Fig. 7*C* shows the rate and extent of

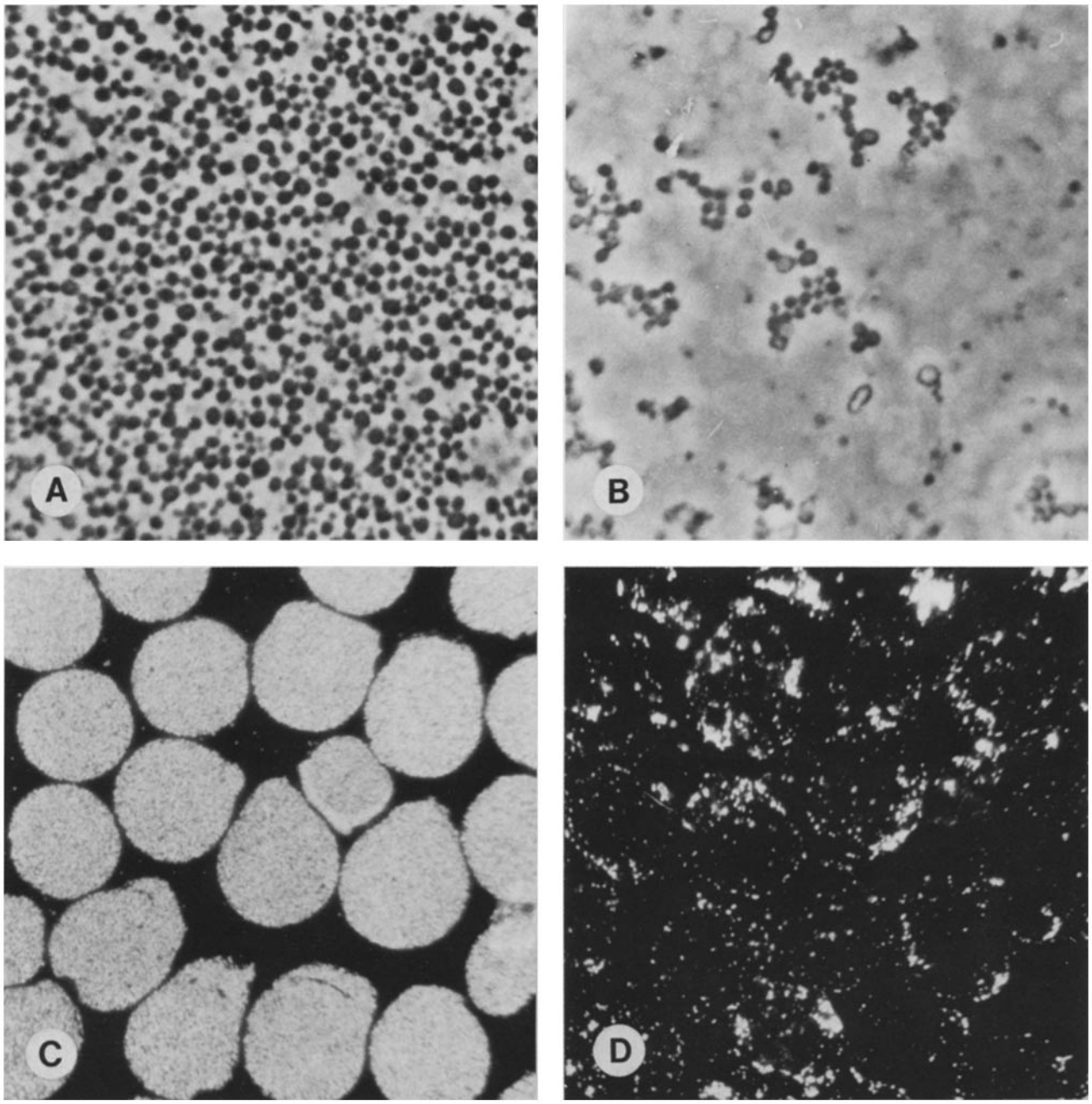

FIGURE 5 Exocytosis of cortical vesicles in planar cortices after perfusion of 17 μM Ca^{++}: comparison of phase-contrast microscopy to light scattering microscopy. Dejellied eggs were allowed to settle on poly-L-lysine-coated coverslips for 5 min. Attached eggs were then gently washed with calcium-free seawater and next sheared with a jet of cortex medium (250 mM potassium gluconate, 250 mM N-methyl-D-glucamine, 180 mM acetic acid, 50 mM PIPES, 5 mM NaCl, and 5 mM EDTA, pH 6.7). Cortices were then treated for 2 min with cortex medium containing either 0.1 μM Ca^{++} or 17 μM Ca^{++}. Next, cortices were transferred to a microscope slide and placed on a microscope stage. (*A*) 0.1 μM Ca^{++}, × 2,000 phase contrast. (*B*) 17 μM Ca^{++}, × 2,000 phase contrast. (C) 0.1 μM Ca^{++}, × 200 light scattering. (*D*) 17 μM Ca^{++}, × 200 light scattering.

exocytosis as a function of time after egg lysis. We chose a 2-min delay after shearing for our subsequent perfusion experiments to minimize this effect (32). In experiments not involving the chamber, calcium was added directly after shearing (Fig. 5).

Once exocytosis had begun we stopped it by calcium removal. Fig. 8*A* shows such an experiment. Isolated planar cortices were first exposed to 17 μM Ca^{++}, and exocytosis began as expected. After 10 s, calcium was removed by perfusion with 10 mM EDTA cortex medium. Exocytosis immediately ceased. The same preparation continued to react when calcium perfusion was allowed to occur again.

Permeability of Cortical Vesicles

Using this cortex preparation, the effects of hyperosmotic media on exocytosis were then studied. An osmotic response requires a membrane permeable to solvent (water) but not solute. To test an osmotic requirement for exocytosis, we first determined the permeability of the cortical vesicle to nonelec-

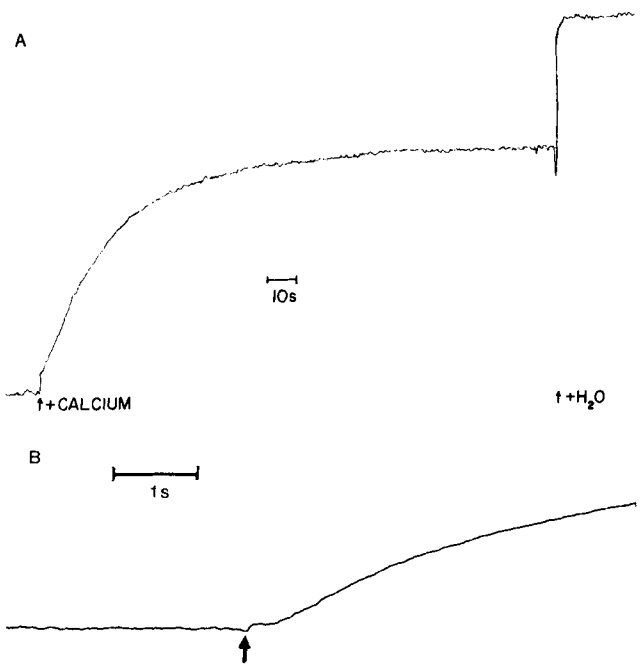

FIGURE 6 (*A*) Cortices were prepared as above and immediately transferred to a perfusion chamber containing 0.1 μM Ca^{++} cortex media and viewed with 100× light scattering microscopy. After focusing, light was deflected through the camera tube to the light detection system described in the text. 2 min after shearing, at the arrow marked +CALCIUM cortices were perfused with 17 μM Ca^{++} media. The resulting change in scattered light amplitude was measured. At the arrow marked $+H_2O$, distilled water was perfused through the chamber to burst remaining granules and obtain the background. (*B*) Cortices were treated as in *A* but only the initial response to calcium is shown with a fast time-response chart recorder. The arrow refers to calcium addition. The time of calcium addition is detected by a temperature transient in the other channel of the recorder (not shown).

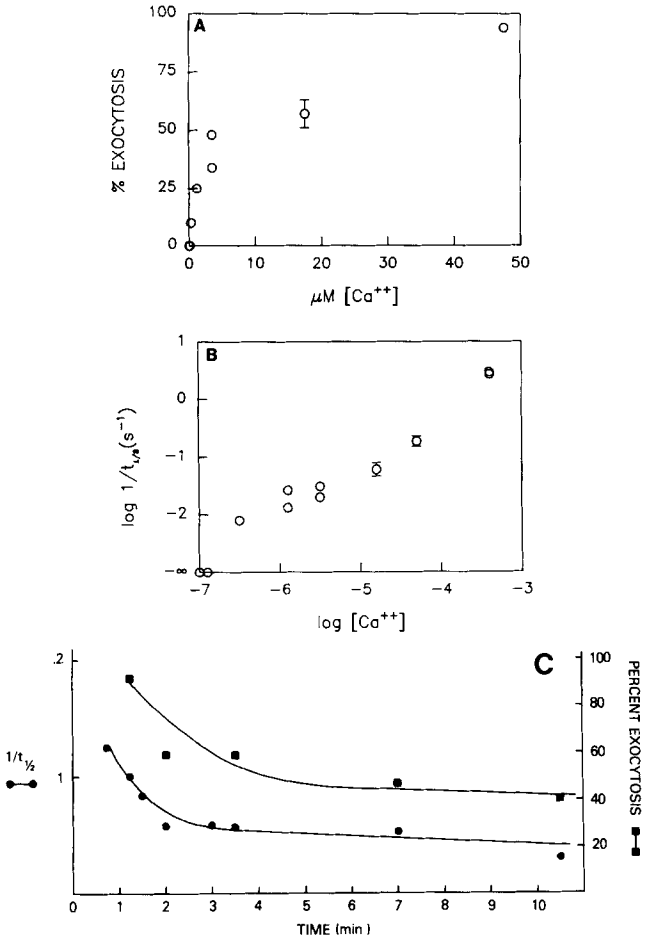

FIGURE 7 The extent and rate of exocytosis was a function of calcium concentration. Cortices were prepared with cortex medium, transferred to a perfusion chamber, and viewed under 100× light scattering microscopy. After focusing, the scattered light intensity was recorded as a function of time. 2 min after shearing, exocytosis was triggered by perfusion of medium containing various concentrations of calcium. Distilled water was perfused through after no further changes were detected (plateau), bursting remaining vesicles. (*A*) The percent exocytosis was determined by dividing the calcium-induced plateau value of light intensity by the difference between the initial light intensity and the water-revealed background. (*B*) The half-time ($t_{1/2}$) was determined by estimating the time between calcium perfusion and half-maximal light scattering change. (*C*) Cortices were perfused with cortex medium containing 17 μM Ca^{++} at various times after shearing, and the extent and half-times measured as above.

trolytes by exposing vesicles to isoosmotic solutions of the solute in question. Since the water activity is identical inside and outside of the vesicle, initially there will be no net movement of water across the vesicular membrane. If the membrane is impermeable to the solute, there will be no changes in volume, and the vesicle will remain intact. However, if the membrane is permeable to solute, that solute will diffuse down its concentration gradient into the vesicle, and water will follow. This water movement, however, will dilute the internal solute, re-establishing a solute gradient, so solute will continuously diffuse into the vesicle, followed by water movement, and so on until the vesicle bursts.

Replacing cortex media with an isoosmotic sucrose solution (1 M sucrose, 2 mM MgCl, 10 mM EGTA pH 6.7) led to slow disappearance of cortical vesicles over minutes. Identical results were obtained with similar isoosmotic solutions of glycerol, urea, and glucose, with the rate of vesicle disappearance a monotonically decreasing function of the nonelectrolyte's molecular weight. These results indicate that the vesicles are permeable to certain nonelectrolytes, including sucrose.

One may worry that this sucrose permeability, unusual for biological membranes, is due to the removal of ions from the bathing media. Vesicles are permeable to sucrose in the ionic milieu of cortex medium as well. When cortices are exposed to 2 M sucrose-cortex medium for 150 s and then returned to cortex medium, 20% of the vesicles burst. Thus sucrose can permeate cortical vesicles in milieu akin to cytoplasm.

Hyperosmotic Solutions

Different results were predicted and seen when the vesicles were bathed in hyperosmotic media. Since the vesicles are permeable to water and sucrose, the prediction is that under hyperosmotic conditions there will be an initial shrinkage (since water is more permeant than sucrose) and water will therefore leave the vesicle rapidly. Sucrose will then slowly enter the vesicle, and as before cause concomitant water entry, reswelling the vesicle to its original size. Thus one predicts a fast transient shrinkage of the cortical vesicle, followed by a slow swelling to the original volume, but not bursting of vesicles.

The experimental observation is that when the isolated cortices are exposed to cortex medium made hyperosmotic with sucrose, no bursting of vesicles is seen for up to 1 h, the longest time observed. Under phase-contrast microscopy, an initial shrinkage is seen by 5 s after perfusion of the hypertonic solution. At 30 s the shrinkage is less marked, but still detectable (Fig. 13*B* [phase photograph] and Fig. 14*B* [digitized data]).

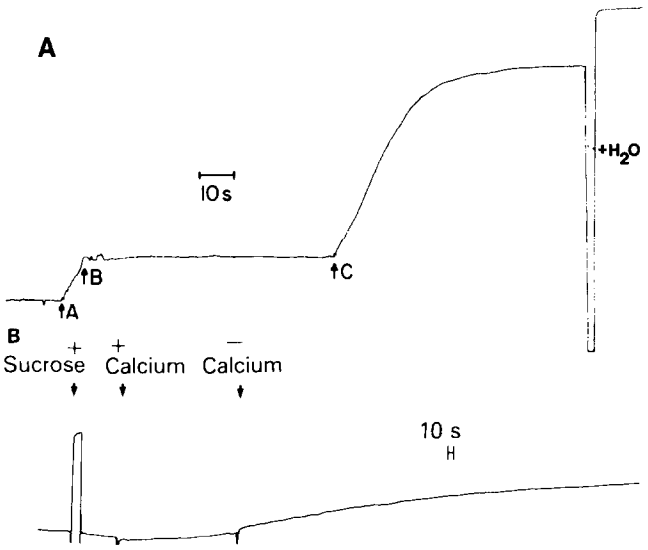

FIGURE 8 Removal of calcium halts exocytosis in isotonic media but not in hyperosmotic media. Cortices were prepared with cortex medium, transferred to a perfusion chamber, and viewed under 100× dark field microscopy. After focusing, the scattered light was recorded as a function of time. (*A*) Isotonic medium. 2 min after shearing, 17 μM Ca^{++} cortex medium was perfused through *A*. After 6 s, 0.1 μM Ca^{++} cortex medium was perfused through *B*. After an additional 75 s, 17 μM^{++} was again perfused through *C*. (*B*) Hyperosmotic medium containing 0.1 μM^{++} was added (+ *Sucrose*). The incident light level was increased, and 160 s later a 4.8 Osm sucrose-cortex medium containing 17 μM Ca^{++} was perfused through (+ *Calcium*). 100 s later, calcium was removed by perfusing through 8 ml of 4.8 Osm sucrose-cortex medium containing 10 mM EDTA, 0.1 μM Ca^{++} (−*Calcium*).

Calcium and Hyperosmotic Solutions

To test the effect of hyperosmotic conditions on calcium-triggered exocytosis, we perfused cortices with sucrose-cortex media of varying osmotic pressures and then exposed them to 17 μM Ca^{++} in that same solution (Fig. 9). As seen in Fig. 9*B*, osmotic pressures greater than 4 Osm/kg dramatically retarded the rate of exocytosis. For example, the half-time for 17 μM Ca^{++}, 2.0 M sucrose-cortex medium (5.34 Osm) was 196 s, as compared with 17 s in the isoosmotic condition. The following observation indicates that the effect of osmotic pressure is after the Ca^{++}-dependent step. If we returned cortices to isoosmotic cortex media 30 s after exposure to 17 μM Ca^{++} in the 2 M sucrose-cortex media, all vesicles immediately disappeared (Fig. 10), despite the presence of 10 mM EDTA. This contrasts to 20% vesicular disappearance upon return to isotonic after exposure to 2 M sucrose cortex-media for the same period of time without calcium.

In contrast, the removal of calcium in the continued presence of sucrose has no effect on the rate of exocytosis; this can be seen in Fig. 8*B*. Cortices are first pretreated with 1.8 M sucrose cortex medium, then 1.8 M sucrose cortex medium containing 17 μM Ca^{++}. After 30 s, we removed calcium by perfusion with 10 mM EDTA in 1.8 M sucrose-cortex medium. In contradistinction to the isoosmotic case (Fig. 8*A*), where exocytosis halted immediately after calcium removal, in the hyperosmotic case exocytosis continued after calcium withdrawal (Fig. 8*B*). Thus calcium exerted an irreversible effect upon the cortex, at a time before the action of sucrose. Note that we withdrew calcium well after its effect would have been complete in isotonic media.

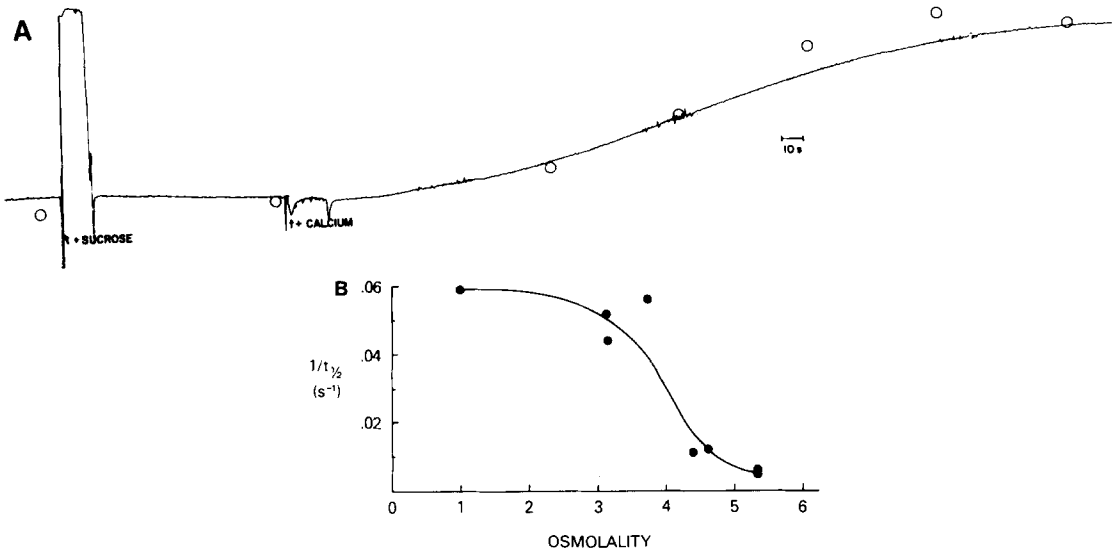

FIGURE 9 Hyperosmotic pretreatment slows exocytosis. Cortices were prepared with cortex medium, transferred to a perfusion chamber, and viewed under 100× light scattering microscopy. After focusing, the scattered light intensity was recorded as a function of time. (*A*) 2 min after shearing, a 5.3 Osm sucrose-cortex medium containing 0.1 μM Ca^{++} was added at the first arrow (+*sucrose*). The incident level was increased. 100 s later, a 5.3 Osm sucrose-cortex medium containing 17 μM Ca^{++} was perfused through (+*calcium*). The continuous line records the light scattering change. Unfilled circles represent the fraction of cortical granules fusing as determined by phase contrast microscopy (1,000×) in a similar experiment. (*B*) A number of cortices were treated as above in *A*. Osmolality was varied with sucrose; Ca^{++} was held constant at 17 μM as estimated with a calcium selective electrode. The curve is drawn by eye for convenience and denotes no theoretical curve.

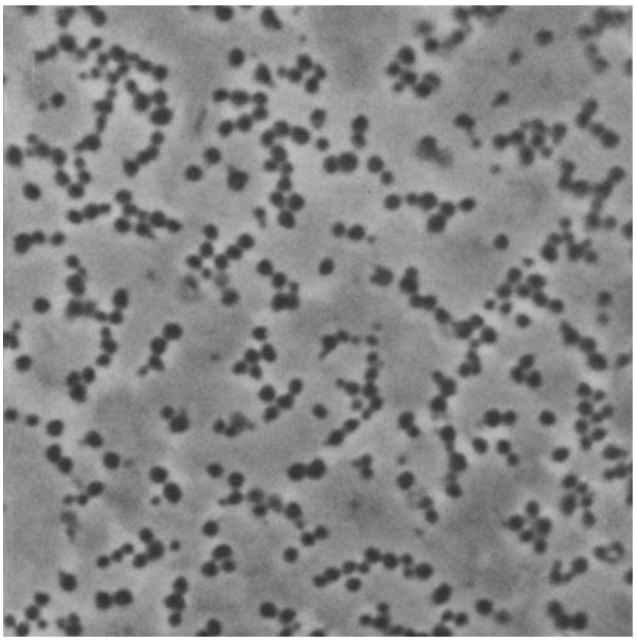

FIGURE 10 Removal of sucrose after calcium treatment leads to immediate exocytosis. Cortices were prepared with cortex medium, transferred to a perfusion chamber, and viewed under 1,000× phase contrast microscopy. 2 min after shearing, 4.8 Osm sucrose-cortex medium containing 0.1 μM Ca^{++} was perfused through. 30 s later 4.8 Osm sucrose-cortex medium containing 17 μM Ca^{++} was perfused through. After an additional 20 s, cortex medium (1 Osm, 0.1 μM Ca^{++}) was perfused through. The cortices were immediately photographed. × 2,000.

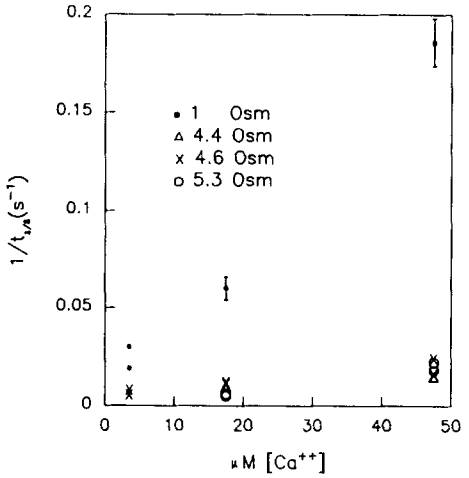

FIGURE 11 Calcium cannot reverse the retardation effected by hyperosmotic milieu. Cortices were prepared with cortex medium, transferred to a perfusion chamber, and viewed under 100× light scattering microscopy. After focusing, the scattered light intensity was recorded as a function of time. 2 min after shearing, media containing added sucrose with varying osmolalities, all with 0.1 μM Ca^{++}, were perfused through. After this hyperosmotic pretreatment, cortices were perfused by media containing identical sucrose as the pretreatment but varying calcium concentrations. Half-times were determined directly from the recording of scattered light.

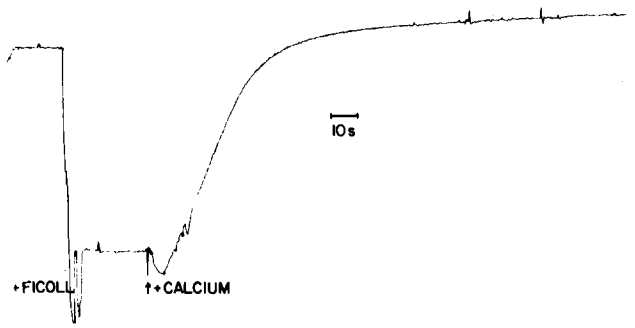

FIGURE 12 Highly viscous solutions do not retard exocytosis. Cortices were prepared with cortex medium, transferred to a perfusion chamber, and viewed under 100× dark field. After focusing, the scattered light intensity was recorded as a function of time. 2 min after shearing, a 30% ficoll-cortex medium containing 0.1 μM Ca^{++} was added. The incident light level was increased. 1 min later (*+calcium*), 30% ficoll-cortex media containing 17 μM Ca^{++} was perfused through.

The fact that exocytosis is retarded but not stopped by hyperosmotic media at 17 μM Ca^{++} suggests that sucrose is not exerting its effect by competing for calcium. To further explore that possibility a number of experiments were performed with differing calcium activities and osmotic pressures (Fig. 11). In contradistinction to the isotonic case, increasing calcium activity did not markedly alter the rate of exocytosis in hypertonic media. Although calcium was required for the reaction, calcium activity was no longer a rate-determining variable.

A number of control experiments were performed to explore any contribution of non-osmotic effects of sucrose. First, viscosity was ruled out by treating cortices at 17 μM Ca^{++} with 30% ficoll (400,000 mol wt) in the medium (Fig. 12). Although the measured viscosity of the ficoll medium was four times that of a 4.4 Osm/kg sucrose medium (96 versus 24 centipoise), exocytosis was only slightly slowed. Second, comparison of results shown in Fig. 9*B* with that expected for a binding isotherm makes unlikely specific inhibition by sucrose or by a contaminant in the sucrose. Third, hyperosmotic sorbitol solutions led to a similar retardation of exocytosis.

We also observed that 5 mM ATP could not reverse the hyperosmotic inhibition, nor did using different media, such as a glutamate-glycine medium which more closely resembles ooplasm (3). We obtained similar results by repeating the hyperosmotic and hyperosmotic plus calcium experiments with isolated cortices of *Lytechinus pictus* eggs.

Calcium Causes Swelling of Vesicles

One prediction of the osmotic hypothesis of exocytosis is that the trigger for exocytosis should induce swelling of cortical vesicles. Since the size of the cortical granule is larger than 0.2 μm, we can directly measure their size with phase-contrast microscopy. With the perfusion chamber described above it is possible to examine the same planar cortex while changing the bathing medium, and record the image either on video tape or film. We could not detect swelling accompanying vesicle exocytosis in isotonic media since discharge of single vesicles was completed within one video frame (17 ms). In hyperosmotic media, however, a calcium-induced swelling of vesicles could be seen over many seconds. The vesicles were photographed at various times in the different media and the vesicular areas measured by image analysis (as described in Materials and Methods) to obtain good statistics.

As seen (Figs. 13 and 14) we detected shrinkage of vesicles 30 s after exposure to calcium. Granule swelling was visible by 80 s. The morphometric analysis of the areas enclosed by

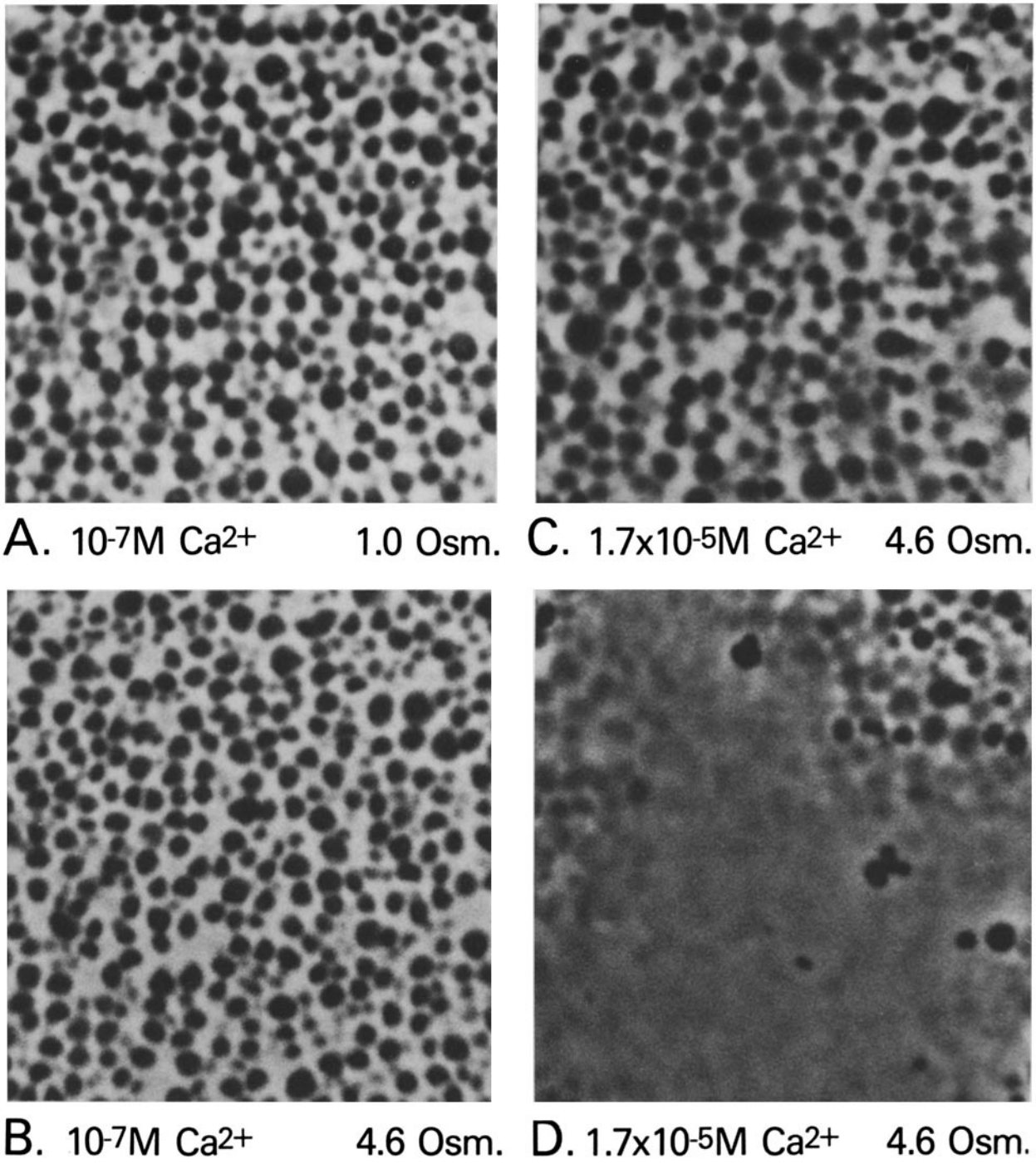

FIGURE 13 Calcium causes cortical vesicle swelling in hyperosmotic media. Cortices were prepared with cortex medium and transferred to a perfusion chamber. 2 min after shearing, cortices were photographed with 1,000× phase contrast microscopy (*A*), and then perfused with 4.6 Osm sucrose-cortex medium containing 0.1 μM Ca^{++}. 30 s later, cortices were again photographed (*B*), and then perfused with 4.6 Osm sucrose-cortex media containing 17 μM Ca^{++}. Cortices were photographed at 80 (*C*) and 113 (*D*) s after addition of calcium. Vesicles appear to shrink with sucrose addition and then to swell to a size larger than their initial size after calcium addition. × 3,400.

the granules showed significant differences (see figure legend) induced by Ca^{++} ($P < 0.001$ for adjacent pairs).

DISCUSSION

Morphological Studies

The electron micrograph studies, utilizing rotary shadow replicas and freeze fracture of rapidly frozen cortices, provided two new views of the cortical vesicle exocytosis. First, the domes seen in whole mounts represent an *en face* complement to the omega figure, previously seen in thin section in this preparation (7, 43). These views, combined with the thin section views and differential interference contrast observations, and the formation of fertilization membrane in vitro in isolated cortices in suspension (33), lead to the conclusion that ultimately the cortical vesicles fuse with the underlying plasma membrane in vitro. Second, freeze-fracture of rapidly frozen cortices showed specialized areas of contact between

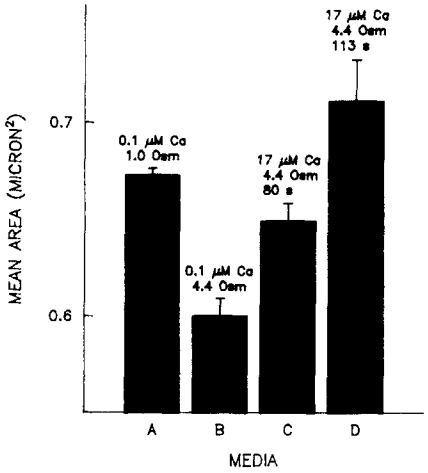

FIGURE 14 A histogram of mean granular area quantifies the swelling caused by calcium. The negatives printed in Fig. 13 were digitized and the areas of 85–312 granules per negative estimated. The probability that the files were the same was <0.0001 for *A* and *B*, 0.0001 for *B* and *C*, 0.0001 for *C* and *D*, 0.0272 for *A* and *D*, and 0.0009 for *B* and *D*.

plasma membrane and cortical vesicles and between cortical vesicles themselves. These areas are free of intramembranous particles and may represent specialized fusion sites noted in fixed material (22). Specialized attachment sites are known also to exist in *Paramecium* involved in trichocyst release (34). The sites between adjacent cortical granules may reflect sites for compound exocytosis. Using video-enhanced asymmetric illumination contrast interference microscopy (19), we have seen compound exocytosis of cortical vesicles in the fertilization reaction in vivo. This intimate contact between membranes contrasts with other well studied cells where fusing vesicles are not in direct contact with the plasma membrane until right before fusion (16).

It is possible that the high degree of curvature in the contact sites is important in concentrating fusagenic lipids. For example, diacyl glyceride might accumulate in these regions.

The Osmotic Hypothesis of Exocytosis

The major result of this study was that two predictions of the osmotic hypothesis of exocytosis were correct: exocytosis is inhibited in vitro by hyperosmotic media, and calcium induces swelling of the exocytotic vesicle. We first demonstrated that the granules are permeable to sucrose. Under hyperosmotic conditions, the addition of calcium results in exocytosis with a dramatically slower rate, and vesicular swelling is slow enough to be seen directly.

Removal of calcium in hyperosmotic solutions, after calcium treatment, did not halt fusion at all. This suggests that the calcium-dependent reaction is early and irreversible. Vesicles need only sufficient swelling to finally fuse.

Removal of a hyperosmotic condition after calcium treatment led to immediate exocytosis. This suggests that calcium increases the osmotic pressure of the secretory vesicle. Vesicles fuse immediately upon swelling.

We imagine that calcium somehow induces an increase in the osmotic pressure of the granule and that water then enters, causing the granule to swell. This increase in osmotic pressure could arise from the activity of hydrolases and proteases in the granule (6, 13), gel hydration (due to loss of cross-linking agents stabilizing the sulfated acid mucopolysaccharides [36]), colloid osmotic swelling due to a change in vesicular membrane permeability, change in activity of insoluble constituents (through mechanisms such as phosphorylation of less hydrophilic sites or binding of Ca^{++} to neutral binding sites), pump activity, and so on. This initial swelling ordinarily leads to tension and fusion. However, in hyperosmotic sucrose this amount of swelling is not sufficient to bring the shrunken vesicles into the taut state required for fusion. Since sucrose is permeable, and there is a large gradient of sucrose, the vesicle slowly swells until reaching the taut state, at which time it fuses. Indeed, when these experiments were repeated with less permeant osmoticants, such as stachyose, exocytosis was prevented and irreversible swelling due to calcium seen (47, 48).

Because the calcium-independent reaction was slowed, it was possible for us to photograph the events that followed sucrose and calcium addition. In the presence of sucrose we detect swelling in response to calcium. Is this swelling an artifact of the hyperosmotic treatment? We found the explosive exocytosis of single granules too fast to characterize in isoosmotic medium. Significant isoosmotic swelling is seen during the cortical reaction of eggs from another echinoderm, the crinoid Comanthus japonica (17), and swelling is also seen in other exocytotic situations (11). It is possible that the same mechanism underlies both calcium-induced swelling of the vesicles and the subsequent dispersal and swelling of the cortical granule contents which eventually lead to the large pervitelline space (24).

Exocytosis of cortical vesicles is inhibited by hyperosmotic conditions in vivo (46, 48), but at much lower osmotic pressures. Here hyperosmotic conditions increase the osmotic pressure of impermeant species in the cytoplasm, and perhaps it is this impermeant osmotic pressure that is preventing exocytosis.

Is osmotic swelling of vesicles related to dispersal of vesicular contents, or to membrane fusion, or to both processes? A recent study of Limulus amoebocytes suggests swelling at the time of release, but it was not possible to discern whether the formation of a pore between the two membranes preceded or antedated the swelling (26). Such pores can be detected by capacitance. Capacitance measurements of sea urchin eggs activated with a calcium ionophore but inhibited by hyperosmotic seawater indicate that pore formation had not occurred (48). An ultrastructural study of this state, calcium-activated yet not fused, may yield pictures of intermediates in exocytosis. The retardation of exocytosis that we see may be caused by a prefusion state that normally has a very short life time, and is more stable without the stress of swelling.

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