High Molecular Weight Polymers Block Cortical Granule Exocytosis in Sea Urchin Eggs at the Level of Granule Matrix Disassembly

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Abstract. Recently, we have shown that high molecular weight polymers inhibit cortical granule exocytosis at total osmolalities only slightly higher than that of sea water (Whitaker, M., and J. Zimmerberg. 1987. *J. Physiol.* 389:527–539). In this study, we visualize the step at which this inhibition occurs. *Lytechinus pictus* and *Strongylocentrotus purpuratus* eggs were exposed to 0.8 M stachyose or 40% (wt/vol) dextran (average molecular mass of 10 kD) in artificial sea water, activated with 60 μM of the calcium ionophore A23187, and then either fixed with glutaraldehyde and embedded or quick-frozen and freeze-fractured. Stachyose (2.6 osmol/kg) appears to inhibit cortical granule exocytosis by eliciting formation of a granule-free zone (GFZ) in the egg cortex which pushes granules away from the plasma membrane thus preventing their fusion. In contrast, 40% dextran (1.58 osmol/kg) does not result in a GFZ and cortical granules undergo fusion. In some specimens, the pores joining granule and plasma membranes are relatively small; in other cases, the exocytotic pocket has been stabilized in an omega configuration and the granule matrix remains intact. These observations suggest that high molecular weight polymers block exocytosis because of their inability to enter the granule matrix: they retard the water entry that is needed for matrix dispersal.

CORTICAL granule exocytosis in sea urchin eggs is initiated at the point of sperm entry and sweeps over the egg surface 15–45 s after insemination (Just, 1919; Anderson, 1968; Eddy and Shapiro, 1976; Chandler and Heuser, 1979). This biologically important event releases enzymes and structural proteins that lead to assembly of the fertilization envelope, an extracellular matrix which prevents polyspermy and protects the early embryo (Endo, 1961; Veron et al., 1977; Chandler and Heuser, 1980*a*; Kay and Shapiro, 1985). Cortical granule exocytosis also serves as an excellent model system for studying the mechanisms of exocytosis. Granule–plasma membrane fusion is initiated by micromolar levels of calcium both in intact eggs and in isolated egg cortices (Steinhardt et al., 1977; Whitaker and Baker, 1983; Zimmerberg and Whitaker, 1985; Swann and Whitaker, 1986).

Recently, it has been shown that hyperosmotic sea water and sea water containing high molecular weight polymers inhibit cortical granule exocytosis in intact eggs as well as in isolated egg cortices (Zimmerberg and Whitaker, 1985; Whitaker and Zimmerberg, 1987). Low molecular weight salts and sugars, however, inhibit at a different stage than do high molecular weight polymers (e.g., dextran). Membrane capacitance measurements in intact eggs show that low molecular weight osmoticants block exocytosis before granule fusion, while dextran polymers block at a point after fusion (Whitaker and Zimmerberg, 1987). To interpret these observations mechanistically, we have determined by electron microscopy the exact point at which exocytosis is arrested. The ultrastructural data we present here indicate that granule fusion in hyperosmotic sea water is prevented by formation of granule-free zone (GFZ)[1] at the cortex that separates the cortical granules from the plasma membrane. The few granules that do fuse with the plasma membrane are arrested at early stages of pore formation that have not been previously visualized. In contrast, polymer (10-kD dextran) solutions do not block granule–plasma membrane fusion. Instead, exocytotic pockets are wide open but contain undischarged granule matrix cores. This suggests that dextran polymers retard water entry into the matrix thus preventing proper dispersal of granule components. These observations allow us to separate cortical granule exocytosis, normally a single continuous process, into four consecutive, but distinct, steps.

Materials and Methods

Lytechinus pictus and *Strongylocentrotus purpuratus*, obtained commercially (Marinus, Inc., Long Beach, CA), were kept at 12°C in aquaria filled with sea water prepared from sea salts (Tropic Marin; Dr. Biener GmbH, Wartenberg, West Germany). Shedding of gametes was induced by injecting

1. *Abbreviations used in this paper*: ASW, artificial sea water; GFZ, granule-free zone; 0Ca0MgASW, divalent cation-free artificial sea water.

Table I. Experimental Solutions

Medium	Composition	Osmolality
		osmol/kg
Stachyose	0.8 M stachyose in ASW	2.60
30% dextran	300 g/liter in ASW	1.30
0Ca0Mg dextran	300 g/liter in 0Ca0MgASW	1.24
40% dextran	400 g/liter in ASW	1.58
Isoosmotic dextran	714 g/kg in 5 mM Hepes	1.04
0.77 M sucrose	0.77 M sucrose in 5 mM Hepes	1.00
1.14 M sucrose	1.14 M sucrose in 5 mM Hepes	1.72

1 ml of 0.5 M KCl into the body cavity and collecting eggs in sea water. Eggs were dejellied either mechanically by three passages through a 90- (*L. pictus*) or 150-μm (*S. purpuratus*) mesh nylon cloth or by suspension in acidified sea water (pH 5 for 2 min). The eggs were then washed twice, resuspended in artificial sea water (ASW; see below) at a cell density of 100 μl packed eggs/ml, and maintained at 16°C until use.

Experiments used ASW of the following composition (in mM/liter): 450 NaCl, 50 $MgCl_2$, 11 $CaCl_2$, 5 $NaHCO_3$, 10 KCl, 1 EDTA, pH 8, at 1.02 osmol/kg. Divalent cation-free sea water (0Ca0MgASW) contained (in mM/liter) 500 NaCl, 10 KCl, 5 $NaHCO_3$, 5 sodium Hepes, pH 8.02, at 0.96 osmol/kg. Osmoticants used were stachyose (Sigma Chemical Co., St. Louis, MO), a 10-kD dextran (T10; Pharmacia Fine Chemicals, Piscataway, NJ), and sucrose (grade I; Sigma Chemical Co.). Osmoticant solutions, as specified in Table I, were prepared either from ASW, 0Ca0MgASW, or distilled water containing 5 mM sodium Hepes, pH 8. Osmotic strength was measured on a vapor phase osmometer (Wescor Inc., Logan, UT) using NaCl standards. The calcium ionophore A23187 (Sigma Chemical Co.) was added as a stock solution in DMSO; the final DMSO concentration (1.25%) had no affect on exocytosis (Whitaker and Zimmerberg, 1987).

Experiments followed a standard protocol. A 250-μl aliquot of the stock egg suspension was hand centrifuged, the supernatant was removed, and the eggs were resuspended by addition of 175 μl of a stachyose/dextran solution with thorough mixing. After a 5-min preincubation at 22°C, 200 μl of test solution containing the calcium ionophore A23187 was added with mixing and the eggs were incubated 5 min at 22°C. The ionophore was mixed into the test solution just before addition, and its final concentration in the egg suspension was 60 μM. Inherent in the standard protocol was presence of a small amount of residual sea water in the test medium (~3% [vol/vol]) carried in with the packed egg pellet. Osmotic strengths have not been corrected for this minor contamination. Unactivated eggs were treated in an identical manner, except for the absence of the calcium ionophore A23187. Control eggs (exposed only to ASW) were also treated identically, except for the absence of stachyose or dextran. After incubation, eggs were either fixed with glutaraldehyde or quick-frozen.

Fixation was carried out by addition of an equal volume of stachyose/dextran test solution containing 4% glutaraldehyde. After fixation for 1 h at 22°C and overnight at 4°C, eggs were washed four times in ASW and then postfixed in diluted ASW (75% of normal tonicity) containing 1% osmium tetroxide. The specimens were then washed in 50 mM sodium acetate buffer, pH 5, and stained en bloc in uranyl acetate (1% in sodium acetate buffer) for 1 h in the dark. After dehydration in graded ethanol solutions, eggs were embedded in Spurr's epoxy resin and silver sections were cut, stained with uranyl acetate and bismuth subnitrate, and viewed at 80 kV in an electron microscope (300; Philips Electronic Instruments, Inc., Mahwah, NJ).

Eggs to be prepared for freeze-fracture were treated with stachyose or dextran solutions by the standard protocol and either fixed with glutaraldehyde as described above or quick-frozen. Cryoprotection of chemically fixed eggs was achieved by adding glycerol dropwise with stirring directly to the glutaraldehyde-containing polymer solution to reach a final glycerol concentration of 20% (vol/vol). After 30 min, the eggs were pelleted in a microcentrifuge (Eppendorf; Brinkmann Instruments Co., Westbury, NY) for 1–2 min at 10,000 *g*, and a drop of the pellet was applied to a gold–nickel specimen carrier and frozen in resolidifying Freon 22. The specimens were then fractured in a freeze-etch unit (400D; Balzers S. p. A., Milan, Italy) at −110°C, allowed to etch for 15 s, and replicated with platinum–carbon from an electron beam gun at a 45° angle. Replicas were cleaned with sodium hypochlorite and viewed in the electron microscope (300; Philips Electronic Instruments, Inc.).

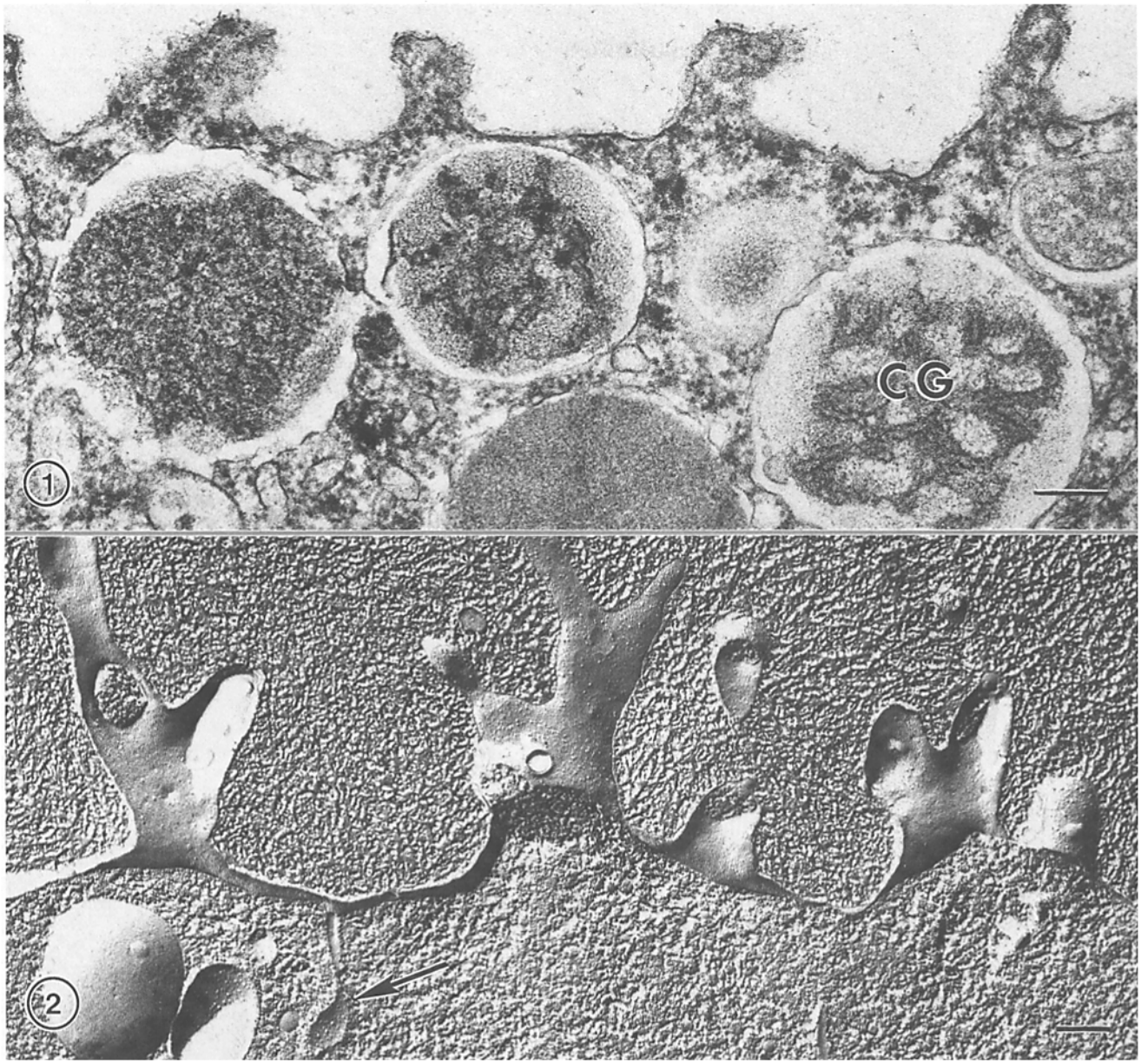
Eggs to be quick-frozen were exposed to stachyose or dextran solutions by the standard protocol and hand centrifuged in a 400-μl microcentrifuge tube until a compact layer was formed (eggs floated in many test solutions). The eggs were then transferred to filter paper on an aluminum planchet and frozen on contact with a liquid helium–cooled copper block in the freezing machine (Cryopress; Med-Vac, Inc., St. Louis, MO) designed by Heuser (Heuser et al., 1979). Eggs tended to be fragile in some polymer solutions, and the above protocol resulted in a portion of cells being damaged. We avoided such damage in later experiments by transferring eggs through polymer solutions as a monolayer adhering to polylysine-coated coverglasses. Coverglasses were then positioned on a drop of ultrasound transmission gel (Lectrosonic; Burdick Corp., Milton, WI) and quick-frozen as described above. Quick-frozen specimens were fractured and replicated in a manner identical to that for chemically fixed samples.

Results

In normal sea water, *L. pictus* eggs have a single layer of cortical granules just below the plasma membrane (Fig. 1). Virtually all granules lie within 0.2 μm of the plasma membrane and, in some cases, one can see small domains in which granule and plasma membranes are in close approximation. Activation of the egg with the calcium ionophore A23187 results in nearly complete exocytosis of cortical granules within 90 s (data not shown). At 5 min after activation, degranulation is >90% complete (Table II) and exocytotic pockets have flattened out (Fig. 2). In addition, the hallmarks of cortical reorganization after activation are present: microvilli each with a thick base and several fingerlike extensions and endocytic invaginations with narrow necks (Fig. 2, *arrow*) have appeared on the egg surface.

Incubation of eggs in low molecular weight osmoticants, such as 0.8 M stachyose, results in a very different ultrastructural picture. Within 5 min, the majority of cortical granules have been separated from the plasma membrane by a GFZ (Fig. 3, *GFZ*). This zone is from 0.5 to 1 μm thick and contains no formed organelles except occasional tubules of endoplasmic reticulum (Fig. 3, *arrows*). Upon activation with the calcium ionophore A23187, the majority of cortical granules do not fuse with the plasma membrane but remain well separated from the cell surface by the GFZ (Fig. 4). The presence of this zone can not be a fixation artifact (e.g., due to the osmotic strength of the fixative) since such a zone is present in both quick-frozen and glutaraldehyde-fixed specimens. A similar GFZ is formed when *S. purpuratus* eggs are incubated in 0.8 M stachyose (data not shown) or in ASW to which has been added other low molecular weight osmoticants, such as sodium sulfate, sodium Hepes, or sucrose (Merkle, C. J., and D. E. Chandler, unpublished observations).

As a result, 0.8 M stachyose blocks >90% of ionophore-induced granule fusion (Table II). There is still a small minority (~9%) of granules that do fuse, possibly because they remain docked near the plasma membrane. Within this group we see examples of what appears to be a series of early events during exocytosis that have been stabilized by hyperosmotic conditions and then captured by quick-freezing (Fig. 5). In some instances the plasma membrane invaginates toward the granule membrane as if to initiate contact with the granule in a very localized region (Fig. 5 *a*). In other cases, small pores ~20–30 nm in diameter have formed, connecting the granule interior with the extracellular medium (Fig. 5 *b*, *arrow*). Finally, some granules are connected to the cell surface by one or more larger pores (Fig. 5 *c*). These examples suggest that 0.8 M stachyose, in addition to blocking



Figures 1 and 2. (Fig. 1) The cortex of an unactivated *L. pictus* egg in ASW (1.02 osmol/kg). Cortical granules (*CG*) lie just beneath the plasma membrane. Specimen was fixed with glutaraldehyde. Bar, 0.2 μm. (Fig. 2) Freeze-fracture replica of an *L. pictus* egg cortex fixed 5 min after activation. Degranulation is complete and exocytotic pockets have flattened. Each microvillus has several fingerlike branches extending from a thick base. Endocytic pits with narrow necks are present (*arrow*). Bar, 0.1 μm.

granule plasma membrane fusion, must inhibit exocytosis after pore formation as well. Normally, at 5 min after activation, exocytotic pockets are no longer present and microvilli have formed (Fig. 2). In hyperosmotic stachyose, none of these processes have taken their normal course, and granules that have fused remain arrested at stages that should have taken place within 10–30 s after ionophore addition.

Treatment of eggs with dextran solutions results in a quite different series of events. Unactivated eggs incubated in 40% dextran exhibit a relatively normal cortex with cortical granules docked just below the plasma membrane (Fig. 6). One unusual feature is the presence of small blebs (Fig. 6, *arrows*) that appeared in freeze-fracture replicas as protrusions on the P face of the plasma membrane (data not shown). Upon ionophore activation in either 30 or 40% dextran, virtually all cortical granules fuse with the plasma membrane (Table II). However, even 5 min after activation, the exocytotic pockets remain well formed and the encased granule matrix has not been properly discharged (Figs. 7 and 8). Of the granules that have fused, 65% exhibit such a core (Table II). Lack of granule core discharge is seen in both quick-frozen (Fig. 9) and chemically fixed samples (Fig. 8), indicating that chemical fixation has not altered core disassembly. The vitelline layer (Fig. 8, *arrow*) is not elevated or converted to the fertilization envelope in these samples, suggesting that enzymatic activities stored within the cortical granules have not been properly solubilized. In some cases, granules are seen at relatively early stages of exocytosis. In Fig. 10, a dim-

*Table II. Effect of Stachyose-, Dextran-, and Sucrose-containing Sea Water on Ionophore-induced Degranulation in Sea Urchin Eggs**

Medium	Unfused granules/ 100 μm of cortex	Fused granules (stabilized)/ 100 μm of cortex		Cores in perivitelline space/ 100 μm of cortex
		Total	With cores	
ASW control	93 ± 8	0	0	0
ASW + A23187	8 ± 5	0	0	0
Stachyose control	100 ± 17	1 ± 2	0	0
Stachyose + A23187	100 ± 23	10 ± 7	0	0
40% dextran control	87 ± 7	0	0	0
40% dextran + A23187	9 ± 5	82 ± 5	53 ± 26	1 ± 2
30% dextran + A23187	1 ± 1	88 ± 4	58 ± 8	1 ± 1
0Ca0Mg Dextran + A23187	2 ± 1	2 ± 3	0	20 ± 6
Isoosmotic dextran + A23187	25 ± 21	29 ± 17	20 ± 14	30 ± 31
1.14 M sucrose + A23187	8 ± 10	0	0	0
0.77 M sucrose + A23187	4 ± 5	0	0	0

* *L. pictus* eggs were fixed 5 min after activation with the calcium ionophore A23187 (using the standard protocol in Materials and Methods) and scored for degranulation by projecting images of thin sections at 30,000×. Controls were treated identically, except that they were not exposed to ionophore. For each condition, six to eight eggs from three different experiments were chosen randomly and counted. Cortical granules (fused or unfused) lying within 1 μm of the plasma membrane and exhibiting a cross section of >0.5 μm in diameter were scored. Scoring is expressed as mean ± SD per 100 linear micrometers of egg cortex. Fused cortical granules, both empty pockets and those with cores, were included in the total. Perivitelline granule cores were scored for those found in the perivitelline space not in contact with the egg surface.

ple in the plasma membrane has pushed into the cytoplasm to nearly contact a granule membrane; many exocytotic pockets are joined to the extracellular space by pores ~0.1–0.2 μm in diameter (Fig. 11).

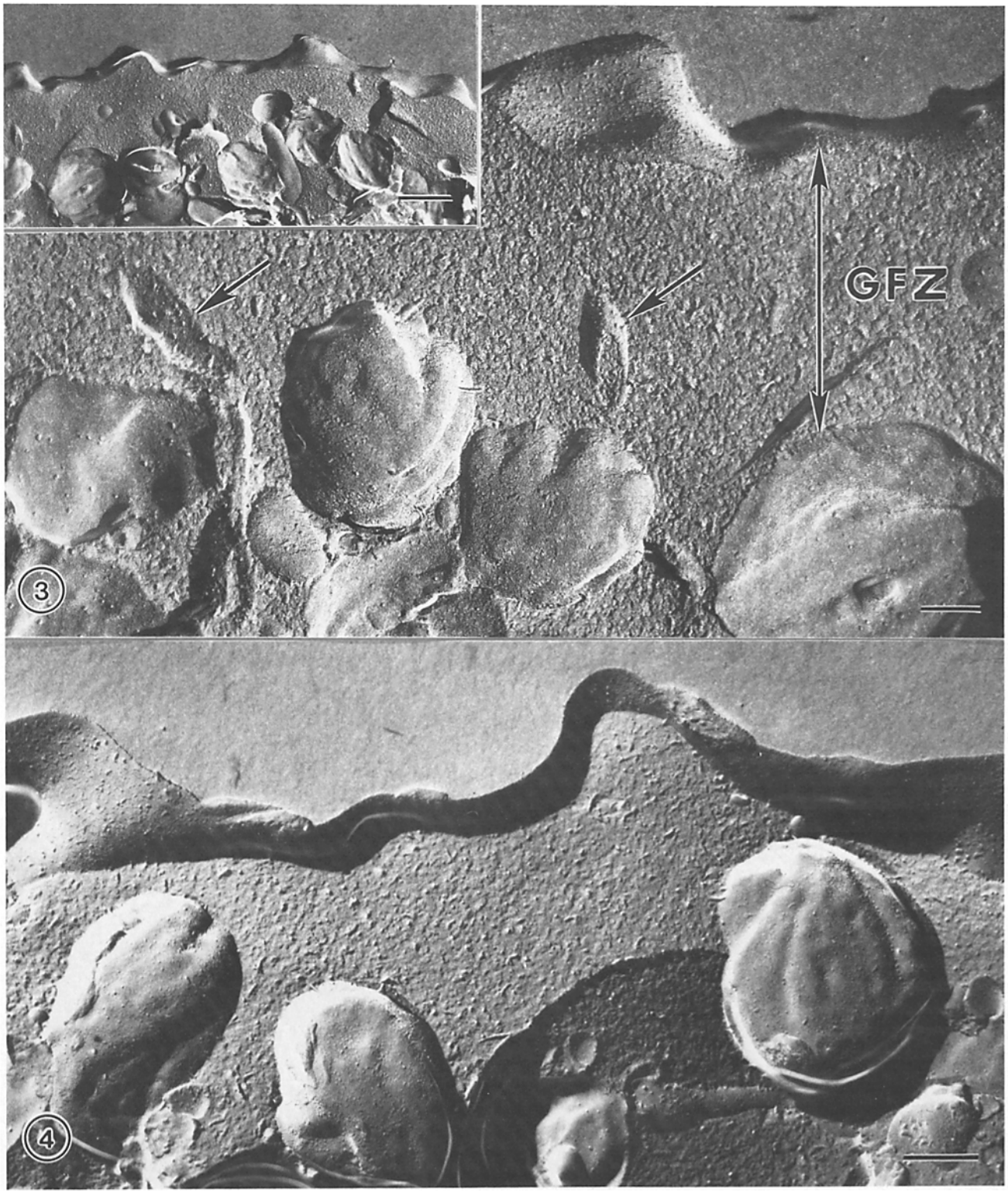
Granule core dispersal can be inhibited in hyperosmotic media containing low molecular weight osmoticants, such as sodium sulfate or sucrose, but such inhibition requires total osmolalities of ≥2.4 osmol/kg (Merkle, C. J., and D. E. Chandler, unpublished observations). The total osmolalities of 30 and 40% dextran solutions are considerably lower than this (Table I), and the portion of the osmotic strength due to added dextran (the colloid osmotic pressure as defined in Whitaker and Zimmerberg [1987]) is lower still, only 0.28 and 0.56 osmol/kg, respectively. Although unlikely, it seemed pertinent to test whether the total osmotic strength of 30 and 40% dextran sea waters could contribute to inhibition of core disassembly. First, eggs were incubated in 1.14 M sucrose (which has a total osmolality slightly >40% dextran sea water [1.72 osmol/kg]) and then activated with the calcium ionophore A23187. In this medium, degranulation occurred normally with complete dissolution of granule cores and flattening of exocytotic pockets (Table II). Similar results were obtained when eggs were activated in 0.77 M sucrose, which is isoosmotic with sea water (Table II). Second, we activated eggs in isoosmotic dextran, which has almost the same osmolality as normal sea water (1.04 osmol/kg). Although the extent of degranulation varied, >65% of granules undergoing fusion showed no evidence of granule core disassembly (Table II). Intact granule cores were present in replicas of quick-frozen eggs (Fig. 12) and in thin sections of glutaraldehyde-fixed eggs (data not shown). Of those cores released from the egg, many were found undissolved in the perivitelline space (Table II). The above results suggest that polymer inhibition of granule matrix dispersal is not due to the modest increase in total osmolality, but is related to the higher molecular mass of the solute instead.

Effective polymer inhibition requires the presence of mono- and divalent cations in the medium (Whitaker and Zimmerberg, 1987). The requirement for divalent cations can be readily demonstrated at the ultrastructural level. Sea urchin eggs were washed in 0Ca0MgASW and resuspended in 0Ca0MgASW containing 30% dextran. These eggs were then activated with the calcium ionophore A23187 using the standard protocol (see Materials and Methods). Table II shows that, 5 min after activation, cortical granule exocytosis is virtually complete. In thin sections (Fig. 13), exocytotic pockets have smoothed out and microvillar growth is underway. A few partially intact cores are seen in the perivitelline space (Fig. 13, *arrow*). In contrast, when divalent cations are present, the granule cores do not dissociate but remain embedded in the exocytotic pockets (Fig. 8 and 9).

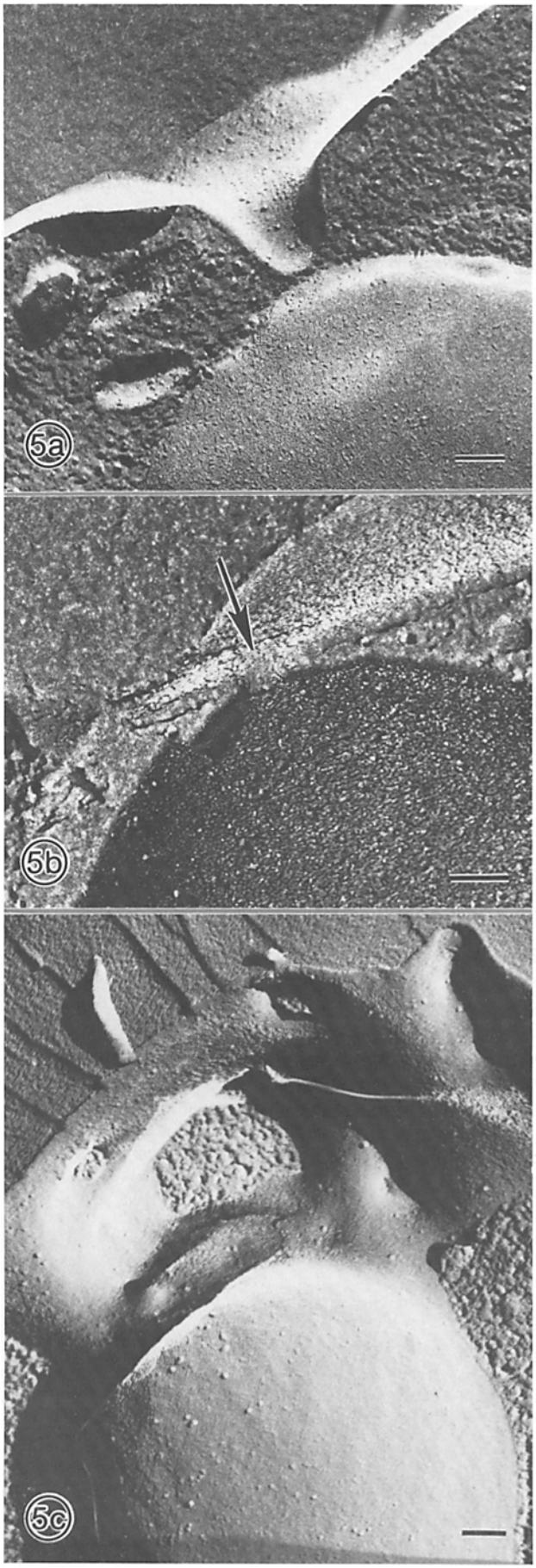
Discussion

A major finding in this study is that high molecular weight osmoticants, such as dextran polymers, inhibit exocytosis in a completely different manner than do low molecular weight osmoticants, such as stachyose, a tetrasaccharide. Low molecular weight solutes cause cytoskeletal rearrangements that result in formation of a GFZ just below the plasma membrane, physically separating cortical granules from their normal association with the plasma membrane. This barrier prevents granule–plasma membrane fusion. Formation of this zone occurs whether the osmoticant is stachyose (this study), sodium sulfate, sodium Hepes, or sucrose, and the extent of granule separation resulting is dependent on osmotic strength (Merkle, C. J., and D. E. Chandler, unpublished observations). Additional study will be required to determine the mechanism of formation, but our present hypothesis is that this zone results from actin polymerization in the cortex, not unlike that seen after fertilization.

The presence of this zone was completely unexpected. We began this study with the thought that hyperosmotic media would halt exocytosis at early stages in fusion/pore formation. Indeed, hyperosmotic media have been shown to slow



Figures 3 and 4. (Fig. 3) Cortex of an *L. pictus* egg quick-frozen in 0.8 M stachyose (2.60 osmol/kg). Cortical granules are separated from the plasma membrane by an organelle-free zone (*GFZ*) ~0.8 μm thick (*inset*). The only formed organelles within this zone are tubules of endoplasmic reticulum (*arrows*). Bars: 0.1 μm; (*inset*) 0.5 μm. (Fig. 4) Cortex of an *L. pictus* egg quick-frozen 5 min after activation in 0.8 M stachyose. Cortical granules have not fused with the plasma membrane. Bar, 0.2 μm.



or block exocytosis in many cell types, including adrenal chromaffin cells, neutrophils, and platelets (Pollard et al., 1977; Hampton and Holz, 1983; Pollard et al., 1984; Kazilek et al., 1988). Likewise, osmotic gradients are known to promote fusion of unilamellar phospholipid vesicles with a planar bilayer. Cohen et al. (1980, 1984) have shown in such a system that calcium induces a prefusion adherence between the vesicular and planar membranes. Membrane fusion follows providing that an osmotic gradient exists between the inside of the vesicle and the *trans* side of the planar membrane. These observations have been interpreted as suggesting that the water flow into secretory granules, before fusion, provides a driving force for membrane fusion and pore formation (Zimmerberg, 1987). It is now clear, in the sea urchin egg, that this interpretation is incorrect. Fusion is prevented not by elimination of the osmotic driving force but rather by granule separation from the plasma membrane.

Even in the face of GFZ formation, a small portion of cortical granules remain docked at the plasma membrane and these are arrested during pore formation. Pores, as small as 20 nm in diameter, were seen in quick-frozen cells exposed to stachyose. These are considerably smaller than any pores seen during cortical granule exocytosis in normal sea water (Chandler, 1984*b*) and are similar in size to pores seen in stimulated mast cells (Chandler and Heuser, 1980*b*; Zimmerberg et al., 1987). They support our contention that exocytosis begins with membrane fusion at one highly localized region and does not require close apposition of granule and plasma membrane over a large area (Chandler, 1988).

We still do not have a good understanding of how pore formation is arrested by low molecular weight osmoticants. An osmotic explanation appears to be ruled out since these osmoticants are small enough to penetrate the granule core after pore formation and therefore could not prevent the inward movement of water. Osmotic effects on granule core swelling, however, might be exerted at the molecular level. The topography of individual core proteins may contain cavities small enough to exclude even low molecular weight solutes (Zimmerberg and Parsagian, 1986). These would then represent sites at which osmotic stress could retard water movements required for conformational changes in core proteins during granule swelling. On the other hand, we can not rule out such indirect effects as an arrest of pore widening by a cortex that has been stiffened by actin polymerization.

In contrast to low molecular weight osmoticants, dextran solutions in sea water do not halt pore widening but do inhibit a subsequent stage in secretion: disassembly of the granule matrix. The small pores formed first have enlarged, and most exocytotic pockets are wide open. Granule cores have not dissociated, and the pockets surrounding them have not become integrated with the cell surface. These data substantiate the conclusion reached by Whitaker and Zimmerberg (1987) that polymer solutions block exocytosis after

Figure 5. Early events in exocytosis captured in *S. purpuratus* eggs quick-frozen 5 min after activation in 0.8 M stachyose. (*a*) An invagination of the plasma membrane comes in close approach to a cortical granule membrane. (*b*) A small pore (*arrow*), 23 nm in diameter, has formed between plasma and granule membrane. (*c*) An exocytotic pocket remains joined to the plasma membrane by two enlarged pores. Bars: (*a* and *b*) 0.05 μm; (*c*) 0.1 μm.

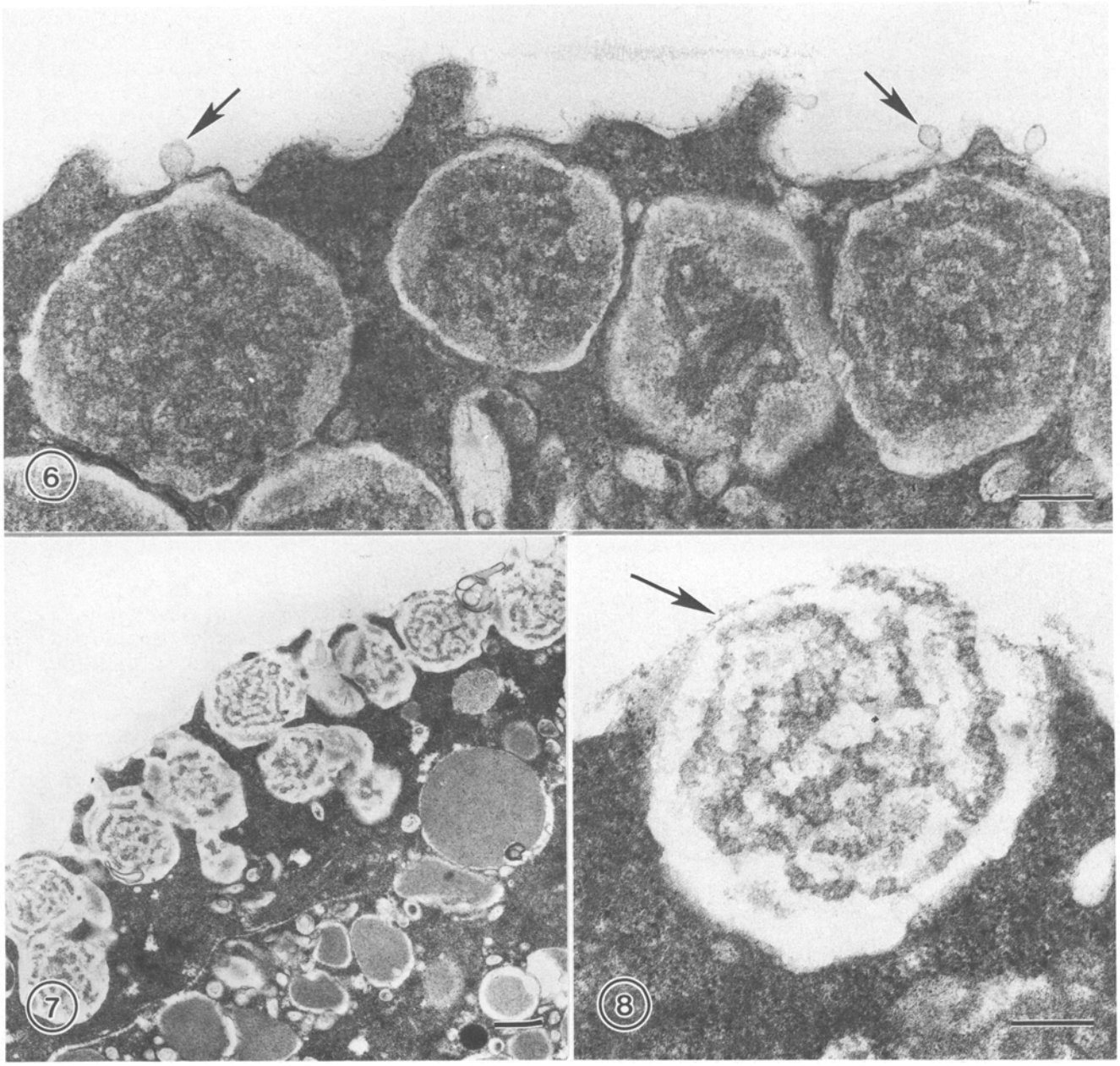
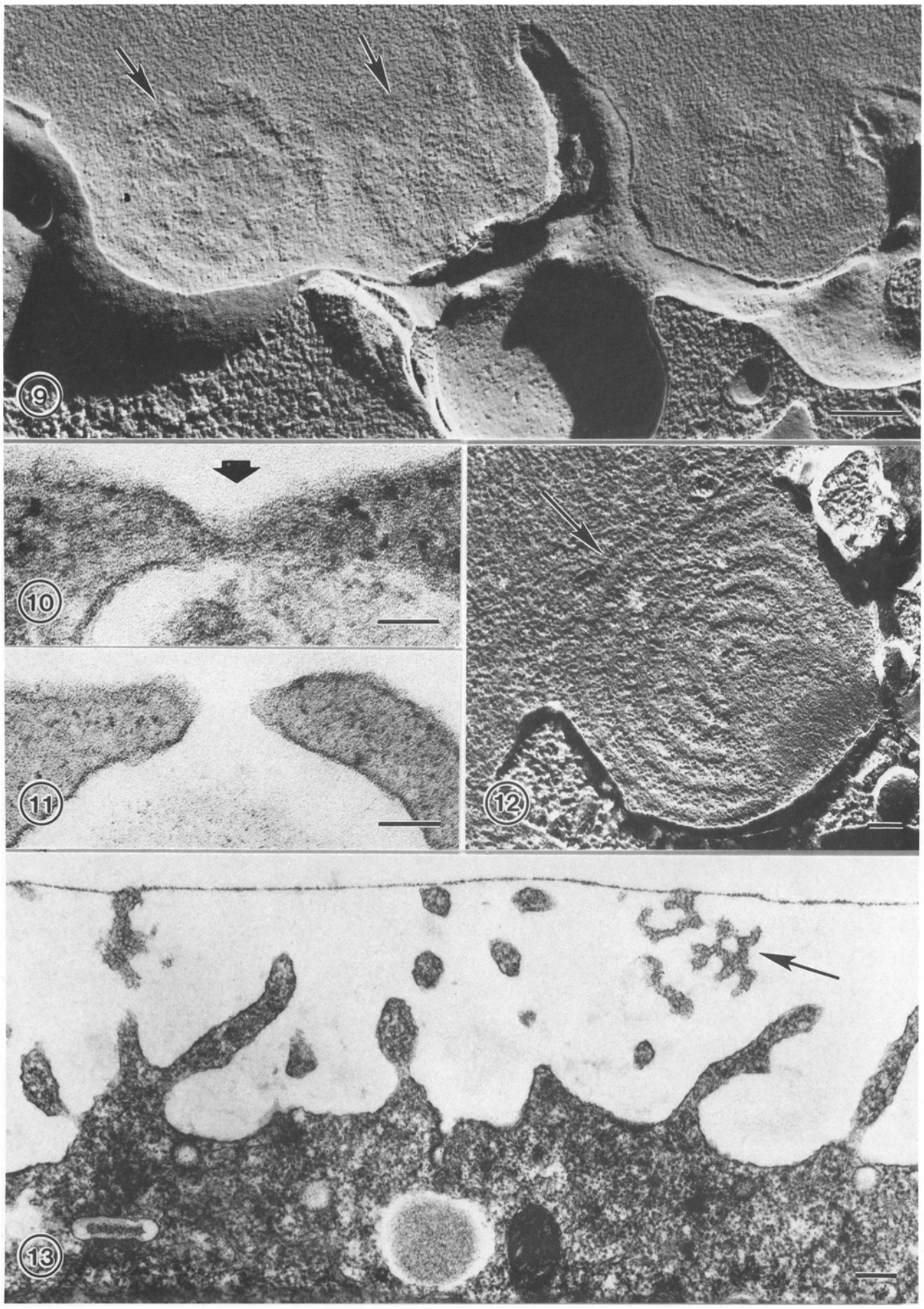


Figure 6–8. (Fig. 6) Cortex of an *L. pictus* egg after a 5-min incubation in 40% dextran sea water. Cortical granules are positioned normally just below the plasma membrane. Small blebs are seen on the cell surface (*arrows*). Specimen was fixed with glutaraldehyde. Bar, 0.2 μm. (Fig. 7) Cortical granule exocytosis in an *L. pictus* egg fixed 5 min after activation with calcium ionophore in 40% dextran. All cortical granules have fused but exocytotic pockets remain intact and the granule matrix undissociated. Bar, 0.5 μm. (Fig. 8) Exocytotic pocket in an *L. pictus* egg activated in 40% dextran. The pocket encases an undissociated granule core; the vitelline layer (*arrow*) has not elevated. Bar, 0.2 μm.

membrane fusion. The fact that dextran solutions isoosmotic with sea water also block granule matrix dispersal indicates that total osmotic strength itself is not the determining factor in inhibition. Furthermore, since exocytosis is normal and complete in isoosmotic sucrose and in sucrose having an osmotic strength equivalent to 40% dextran ASW, it would appear that the molecular mass of the osmoticant is the important parameter for inhibition.

Whitaker and Zimmerberg (1987), in fact, have shown at the light microscopy level that polymers >3,500 D inhibit matrix dispersal, while those below this figure do not unless very high osmolalities are reached. Our data support their contention that high molecular weight polymers cannot enter the granule matrix and therefore reduce water activity outside the matrix and retard water flow into the granule core during disassembly. Light microscopy of exocytosis in chromaffin cells suggests a similar series of events (Edwards et al., 1984). Upon ionophore stimulation, small blebs of low refractive index, about the size of chromaffin granules, appear transiently on the cell surface. These blebs probably represent granule cores that undergo extrusion and dissolution during exocytosis; like cortical granule cores, they are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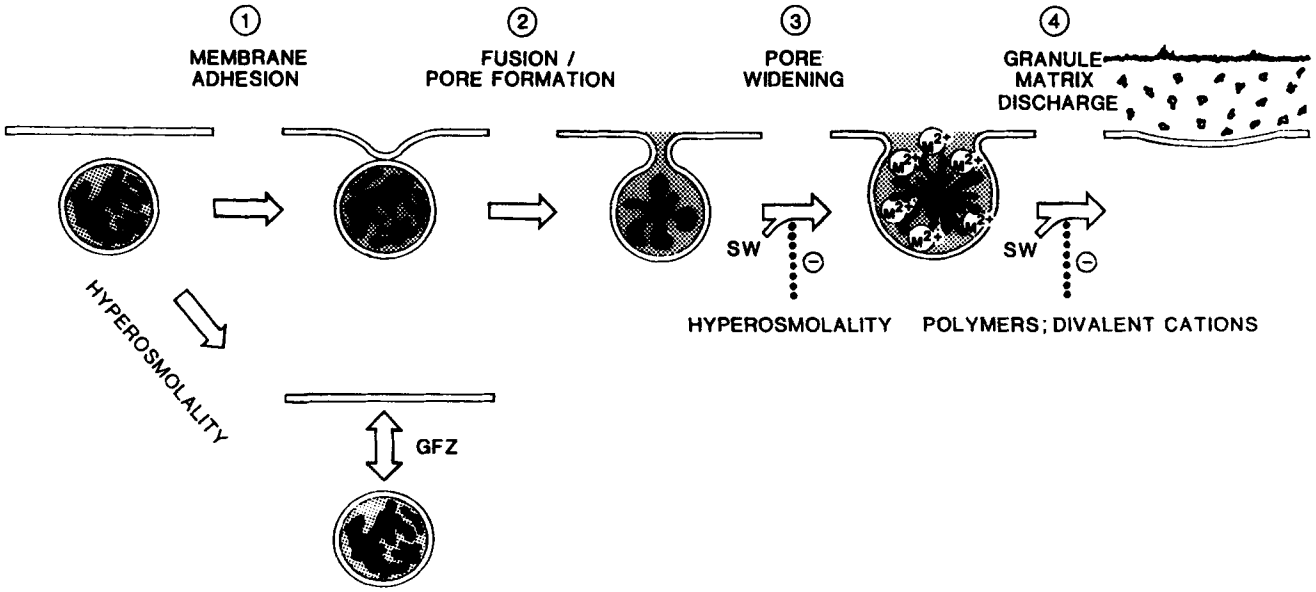

Figure 14. Diagrammatic summary of the four steps in cortical granule exocytosis.

stabilized in the presence of polymers such as Ficol (Edwards, C. D., personal communication).

Inhibition of exocytosis by low molecular weight osmoticants and by high molecular weight dextrans at specific stages allows us to separate the complete process into four steps: membrane adherence, membrane fusion/pore formation, pore widening, and granule matrix discharge (Fig. 14; Zimmerberg, 1987). Membrane adherence is characterized by dimpling of the plasma membrane inward and contact of the two membranes in a highly localized region. Dimpling of the plasma membrane is similar to that described in mast cells (Chandler and Heuser, 1980*b*), amebocytes (Ornberg and Reese, 1981), and, previously, sea urchin eggs (Zimmerberg et al., 1985). Step two, membrane fusion, occurs within the region of adherence through formation of a small pore. Ultrastructural studies in amebocytes (Ornberg and Reese, 1981), mast cells (Chandler and Heuser, 1980*b*; Chandler, 1984*a*), and sea urchin eggs (Chandler, 1984*b*) indicate the presence of pores 10–30 nm in diameter, while electrophysiological studies in mast cells (Zimmerberg et al., 1987) suggest that initial pores may be as small as 1 or 2 nm in diameter. Clearly, pore formation requires membrane adherence since separation of cortical granules from the plasma membrane in hyperosmotic media (>2.0 osmol/kg) blocks granule fusion completely. The third step, pore widening, is also blocked in hyperosmotic media (see Fig. 5, *b* and *c*). Pore widening is slowed by hyperosmotic solutions in beige mouse mast cells as well (Zimmerberg et al., 1987). In this system, electrophysiological measurements show that pore widening is an extremely variable and dynamic process (Curran, M., J. Zimmerberg, and F. S. Cohen, unpublished observations). Additional experiments are needed to discern which cellular elements are responding to osmotic pressure at this step.

In the fourth and final step, the granule matrix must be discharged into the extracellular space. This step appears to require water movement into the matrix since it is inhibited only by polymers which, due to their exclusion, selectively reduce water activity outside of the matrix. Dispersal is also retarded by the presence of divalent cations; one possibility is that calcium and/or magnesium ions help bind the matrix together by charge interaction with anionic matrix constituents and that calcium must be displaced during matrix discharge. A similar sequence may occur in nematocyst discharge: x-ray microanalysis has shown that calcium is lost rapidly from the cyst matrix during release (Lubbock et al., 1981). This is opposite to the role for charge interactions in secretory granule discharge found in a number of cells. Trichocyst discharge in *Paramecium*, for example, is accompanied by rapid matrix expansion that appears to be due to extracellular calcium displacing phosphate ions that crosslink matrix contents (Bilinski et al., 1981; Gilligan and Satir, 1983). Likewise, swelling of mucopolysaccharides from iso-

Figures 9–13. (Fig. 9) Freeze-fracture replicas reveal similar exocytotic pockets in eggs quick-frozen in 30% dextran 5 min after activation. Etching demonstrates that granule cores are still intact (*arrows*). Bar, 0.2 μm. (Figs. 10 and 11) Early stages of granule–plasma membrane approach and fusion in *L. pictus* eggs fixed 5 min after activation in 40% dextran. (Fig. 10) A dimple in the plasma membrane (*arrow*) extends inward towards the granule membrane. Bar, 0.05 μm. (Fig. 11) Widening of the pore formed during granule–plasma membrane fusion has been arrested by dextran. Bar, 0.1 μm. (Fig. 12) Exocytotic pocket in an *S. purpuratus* egg quick-frozen in isoosmotic dextran 5 min after activation. The granule core (*arrow*) is still intact. Bar, 0.1 μm. (Fig. 13) *L. pictus* eggs activated in 0Ca0MgASW containing 30% dextran exhibit complete degranulation and microvillar growth. Specimen was fixed in glutaraldehyde 5 min after addition of the calcium ionophore A23187. Bar, 0.2 μm.

lated secretory granules is extremely rapid and is triggered by entry of calcium ions into the granule which bind to the granule contents creating repulsive forces that drive content disperal (Verdugo, 1984).

Finally, a rather interesting observation in this study is that exocytotic pockets do not flatten out unless the granule core is discharged; the two processes appear to be coupled. Apparently, the intact core is able to maintain pocket integrity and in doing so halts further cortical reorganization, such as endocytosis and microvillar growth. Again, one might expect that it is hyperosmolality that stabilizes exocytotic pocket architecture since the egg has a high surface-to-volume ratio in this configuration. This does not seem to be the case, however, since flattening of these pockets occurs entirely normally in sucrose solutions of the same osmolality as inhibitory dextran solutions.

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