MEMBRANE FUSION DURING SECRETION

Cortical Granule Exocytosis in Sea Urchin Eggs as Studied by Quick-Freezing and Freeze-Fracture

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ABSTRACT

Exocytosis of cortical granules was observed in sea urchin eggs, either quick-frozen or chemically fixed after exposure to sperm. Fertilization produced a wave of exocytosis that began within 20 s and swept across the egg surface in the following 30 s. The front of this wave was marked by fusion of single granules at well-separated sites. Toward the rear of the wave, granule fusion became so abundant that the egg surface was left with confluent patches of granule membrane. The resulting redundancy of the egg surface was accommodated by elaboration of characteristic branching microvilli, and by an intense burst of coated vesicle formation at ~2 min after insemination.

Freeze-fracture replicas of eggs fixed with glutaraldehyde and soaked in glycerol before freezing displayed forms of granule membrane interaction with the plasma membrane which looked like what other investigators have considered to be intermediates in exocytosis. These were small disks of membrane contact or membrane fusion, which often occurred in multiple sites on one granule and also between adjacent granules. However, such membrane interactions were never found in eggs that were quick-frozen without fixation, or in eggs fixed and frozen without exposure to glycerol. Glycerination of fixed material appeared to be the important variable; more concentrated glycerol produced a greater abundance of such "intermediates." Thus, these structures may be artifacts produced by dehydrating chemically fixed membranes, and may not be directly relevant to the mechanism by which membranes naturally fuse.

KEY WORDS exocytosis · membrane fusion · quick-freezing · fixation and glycerination artifacts · fertilization

Sea urchin eggs contain a single layer of cortical granules just under the plasma membrane, which undergo exocytosis during fertilization (1, 3, 7, 8, 22, 30, 38). This so-called "cortical reaction" occurs in a wave that originates at the point of sperm attachment and travels the entire surface of the egg within 30 s (7, 22, 30, 38). In the exocytotic reaction, the membranes of the cortical granules fuse with the plasma membrane and release enzymes, which in turn cause the vitelline layer to rise away from its normal position close to the egg plasma membrane and become a tough fertilization envelope (1, 3, 8, 22, 38). Cortical granule exocytosis is triggered by an increase in the cyto-

plasmic free calcium concentration (21, 35), which results from a release of intracellularly stored calcium (21, 36). In other eggs, this rise in the intracellular calcium level has been visualized with a calcium-sensitive photoprotein (10) and seen to sweep across the egg just in front of the wave of exocytosis.

This wavelike spread of exocytosis offers an opportunity to determine the sequence of events that occurs during exocytosis, simply by observing in the electron microscope the spatial distribution of the different stages of exocytosis across the egg surface. Early stages of exocytosis will predominate at the front of the wave, while intermediate and later stages will be more abundant at the middle and rear of the wave.

Early stages might be expected to include, first, a close apposition of the granule and plasma membranes, such as was first observed in thin sections of capillary endothelial cells (26), cells specialized for vesicle shuttling. Such close apposition of the two involved membranes has since been found in a wide variety of other cells that carry out exocytosis (6, 18, 19, 20, 23, 25, 26, 37). Membrane contacts have also been recognized in freeze-fracture replicas of secretory cells, where they commonly appear as intramembrane particle (IMP)-free domes in the plasma membrane overlying secretory granules (6, 20, 24). The absence of IMPs on these domes has been held as evidence of depletion of membrane proteins from the zone of impending membrane fusion.

Other intermediate stages of exocytosis might also be expected, though they have been less well substantiated. Palade and Bruns' (26) early study of the capillary also provided micrographs in which it appeared that parts of the apposed vesicle and plasma membranes at the site of the fusion had been eliminated, because only two dark lines remained, which presumably implied one single bilayer membrane. Such a "bilayer diaphragm" has also been seen quite distinctly in one freeze-fracture study of exocytosis in zoospores, where it could be distinguished from simple contact of two adjacent membranes (29).

Freeze-fracture has also revealed that in many protozoa a distinct membrane specialization heralds the exact location where exocytosis will occur. This specialization, termed by the original observers a "rosette," consists of several unusually large IMPs that are arranged in a ring around the spot in the plasma membrane where exocytosis is always found (33). Current evidence suggests these IMPs may be calcium channels in the plasma membrane (32). A similar association of synaptic vesicle exocytotic sites and large IMP's has been observed at the frog neuromuscular junction and at other neuronal synapses (15). So far, however, no other secretory cells have been found to have morphologically identifiable sites for exocytosis.

In this report, quick freezing of sea urchin eggs during exocytosis reveals that membrane fusion does not occur at physically differentiated sites on the plasma membrane and does not appear to proceed through any of the intermediates described above. Furthermore, comparison of eggs quick-frozen versus those frozen by conventional means shows that the traditional soak in antifreezes may produce artifacts which can be confused with intermediates of exocytosis.

MATERIALS AND METHODS

Pacific sea urchins *Strongylocentrotus purpuratus* were obtained either commercially (Pacific Biomarine Supply Co., Venice, Calif.) or courtesy of Dr. Daniel Mazia and kept at 11°C in artificial seawater ("Instant Ocean," Aquarium Systems Inc., Eastlake, Ohio). They were induced to shed eggs or sperm by injecting 0.5 M KCl into their coelem. Shed eggs were cleaned of jelly by passing them through a 150-μm mesh nylon cloth and stirred gently in fresh seawater at 10°C until ready for use. Sperm was collected "dry" and kept at 5°C. 10 min before fertilization, sperm was diluted 1:100 with seawater, and then the eggs and sperm were brought to room temperature for all the experiments.

Fertilization was carried out by mixing 1 ml of a 1% sperm suspension with 6 ml of an egg suspension which would pack down to ~0.1–0.2 ml of eggs on centrifugation. Then, at the appropriate times, the fertilized eggs were fixed by adding an equal volume of 3.7% glutaraldehyde in diluted seawater (80% of normal tonicity). Aldehyde fixation proceeded for 1 h at room temperature, after which the eggs were washed in seawater and processed further.

Eggs for freeze-fracture were next suspended in 30% glycerol-70% seawater for 1.5 h, and packed by centrifugation at 100 *g* for 1 min. A drop of the packed cells was sandwiched between two gold "hats" which were coated with a concentrated solution of polyvinyl alcohol so that they would stick together after freezing (compare with Pauli et al. [27]). Freezing was accomplished by plunging these sandwiches into melting Freon 22 at −150°C. Unetched samples were fractured at −130°C in a Balzers 301 unit (Balzers Corp., Nashua, N. H.), and replication was done with platinum carbon from an electron beam gun mounted at 45°C. Alternatively, samples were fractured at −110°C and allowed to etch 20 s before replication. Replicas were cleaned with methanol and sodium hypochlorite and viewed in a JEM 100B

electron microscope at 80 kV.

Other eggs were postfixed in 1% osmium tetroxide in diluted seawater (75%) for 1.5 h at room temperature, washed in seawater, and block-stained with 1% uranyl acetate in 50 mM Na acetate buffer, pH 5.0, for 1 h in the dark at room temperature. They were dehydrated with graded ethanols and embedded in English Araldite. Silver sections were cut on a Porter-Blum MT-2B ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) and stained with 10% uranyl acetate in 50% methanol, followed by 0.4% lead citrate in 0.15 N NaOH.

Quick-freezing was done with the machine designed by Heuser et al. (14, 16) which utilizes a falling plunger to press samples of tissue or isolated cells against a copper block cooled by liquid helium. A drop of concentrated egg suspension was placed on a fragment of a cover glass previously coated with protamine sulfate. Cover glasses, after careful washing and rinsing, were coated by immersion in 2% cyanamide for 5 min, 0.2% protamine sulfate for 5 min, then both together for 30–60 min. Coated cover glasses were rinsed thoroughly and air dried just before use. Eggs were allowed to settle on the cover glasses for 20–30 min, then fertilized by dipping the cover glasses into a 1% sperm suspension. This dip washed away excess eggs and left a monolayer of fertilized eggs adhering to the cover glass. The cover glass was then placed on a cushion of liver at the plunger tip, and then dropped, by gravity, onto the very cold copper block. Methods for subsequent fracturing and replication of such quick-frozen samples have been described by Heuser et al. (14).

All illustrations of freeze-fracture replicas have been photographically reversed and platinum deposits appear white; the direction of shadowing was from the top or upper left corner of each figure.

RESULTS

Thin sections of sea urchin eggs displayed a single layer of membrane-bounded cortical granules just under the plasma membrane. The granules were easily recognized by their striated contents (Fig. 1*A*). The plasma membrane displayed a regular array of short, stubby microvilli and was uniformly covered by the vitelline layer which appeared in thin sections, such as Fig. 1*A*, as a thin, distinct lamina ~20 nm outside the plasma membrane. In freeze-fracture replicas of quick frozen eggs, the cortical granules were distinct and were always visibly separated from the plasma membrane and from each other (Fig. 1*B*). Microvilli on the plasma membrane usually broke during fracture and left a regular array of what looked like stumps when viewing the P fracture face, as in Figs. 1 and 2. Between these stumps, P-face intramembrane particles appeared to be randomly distributed, both in eggs that had been quick frozen (Fig. 2) and in eggs that had been fixed, glycerinated, and frozen (not shown). E-face particles in egg plasma membranes were fewer and less prominent than in the P face, but were also randomly distributed (Fig. 3). Thus, neither face displayed specialized arrays of intramembrane particles that could represent sites for sperm binding or sites for cortical granule exocytosis.

Previous studies have shown that the first cortical granules begin to discharge at the point of sperm penetration, and that subsequent granules are recruited in a wave that spreads out from that point over the surface of the egg. In light microscopy of thick sections, the first signs of the wave could be seen at 20–30 s after insemination, at which time appeared a localized elevation of the vitelline layer above gaps in the continuous row of dark granules that ring the cell periphery (arrow, Fig. 4*A*).

Between 30 and 60 s, eggs displayed varying stages of propagation of this exocytotic wave, starting with a progressive increase in the abundance of individual exocytotic profiles (arrows, Fig. 4*B*), and proceeding to confluent areas of total granule discharge. By 60 s, most eggs appeared to have discharged all their granules, and their vitelline membranes were completely raised (Fig. 4*C*).

The double-replica freeze-fracture technique yielded particularly expansive views of the egg plasma membrane which often displayed this wave of release as a clear-cut two-dimensional pattern. Such a pattern is seen in Fig. 4*D*, where sites of granule fusion were blackened to make them more visible. This figure illustrates the front of the wave where exocytotic images are few and far between, the middle where they are abundant enough to lie adjacent to each other in many places, and the rear of the wave where exocytotic granules involve the whole area and thus become totally confluent. Higher magnification of the plasma membrane near the front of this wave (Fig. 5*A*) revealed a few isolated exocytotic pockets, but no other major change in the surface from that seen in unfertilized eggs. There was no sign of any vesicles pushing against the surface to form domes, which might herald preliminary points of granule and plasma membrane apposition. Still higher magnification of the egg surface (Fig. 5*B*) revealed that the membrane in the depths of these individual exocytotic pockets displayed a lower density of P-face intramembrane particles than the rest of the plasma membrane, which was expected since the

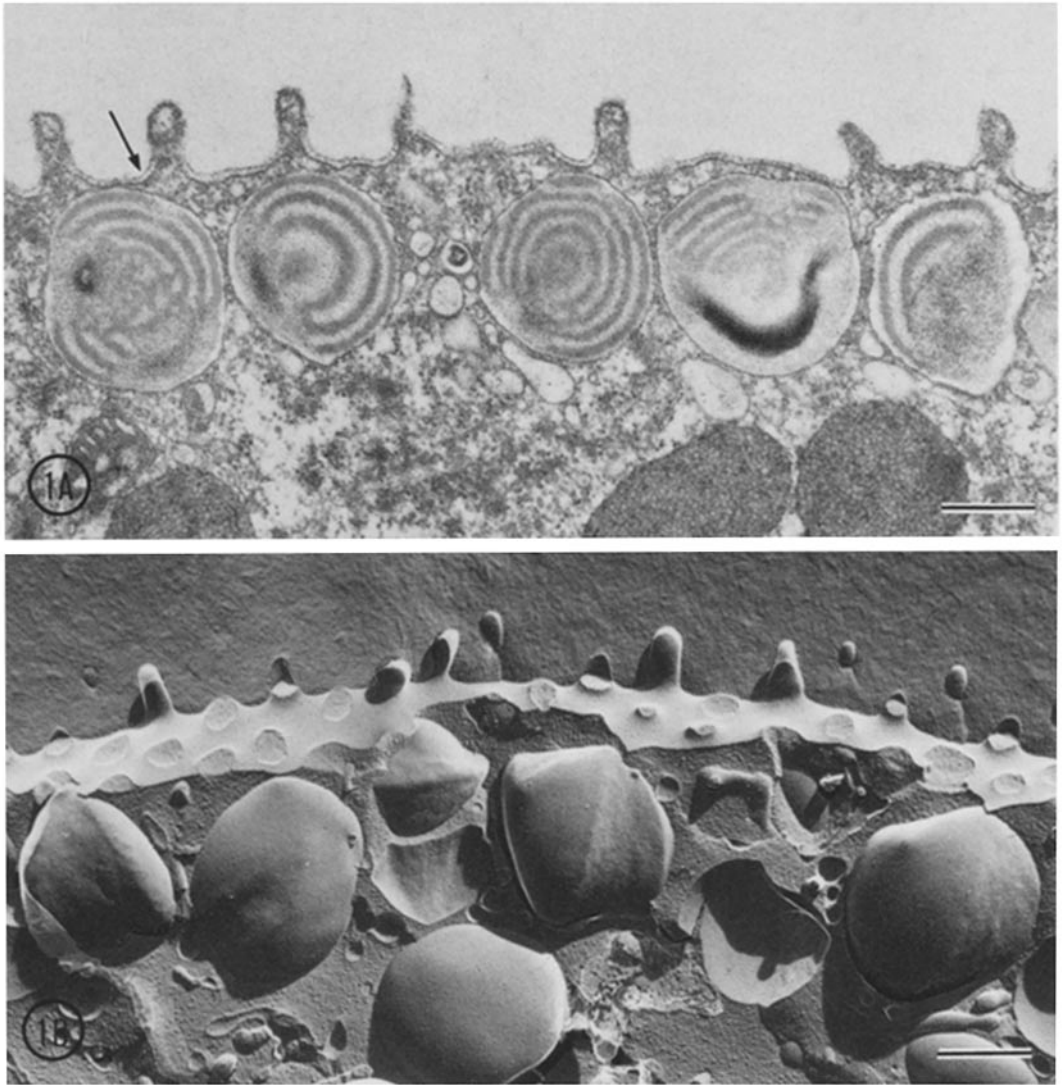

FIGURE 1 (*A*) Electron micrograph of the cortex of an unfertilized sea urchin egg. Cortical granules lie in a single layer just beneath the plasma membrane and have typically striated contents. The vitelline layer is the thin, fuzzy coat just outside the plasma membrane (arrow). The specimen was fixed in glutaraldehyde, postfixed with OsO_4, block-stained in uranyl acetate, and embedded in Araldite. (*B*) Freeze-fracture replica of the cortex of an unfertilized egg. Cortical granules lie just under the P face of the plasma membrane. The specimen was quick frozen. This and all subsequent freeze-fracture plates have been photographically reversed and platinum deposits appear white. Bar, 0.5 μm. × 25,000.

membranes of undischarged cortical granules inside the cell also had a low particle density. But otherwise there was no sign of any change in the random distribution of intramembrane particles in the plasma membrane: there were no clearings or particle aggregates that might indicate where exocytosis was about to occur at the moment the wave was stopped. The only sequence that emerged was that the earlier stages of exocytosis, at the front of a wave and at early times, were relatively narrow openings into underlying granules; and the later stages, which became more prevalent at later times, were more widely open or in various stages of collapse, until eventually flat

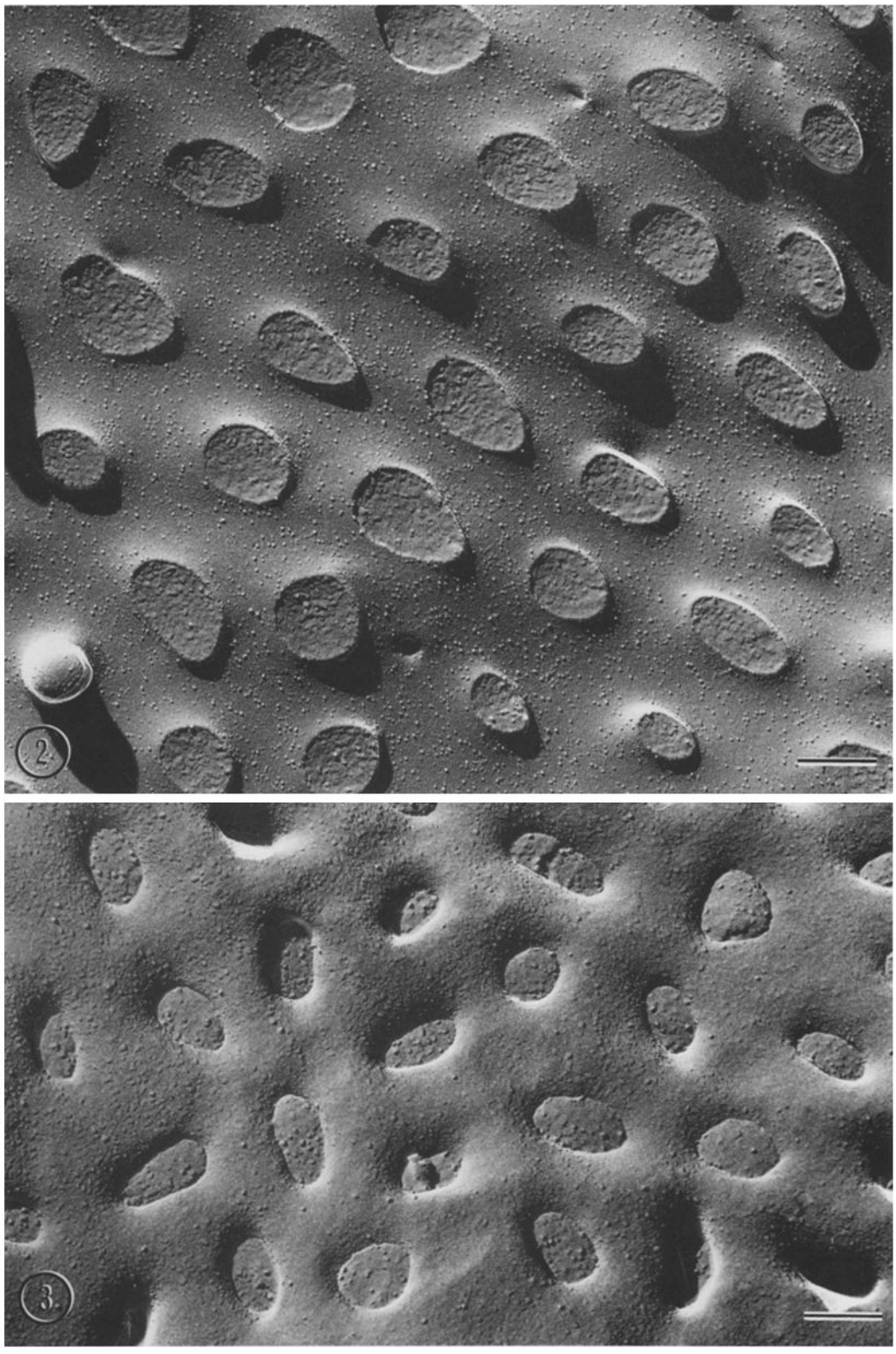

FIGURE 2 Plasma membrane P face of a quick-frozen, unfertilized egg. The stumps of fractured microvilli appear in a regular array; intramembrane particles between microvilli are distributed randomly. Bar, 0.2 μm. × 60,000.

FIGURE 3 Plasma membrane E face of an unfertilized egg that was fixed in glutaraldehyde, glycerinated, and frozen in Freon. Intramembrane particles are less prominent and random in distribution. Bar, 0.2 μm. × 60,000.

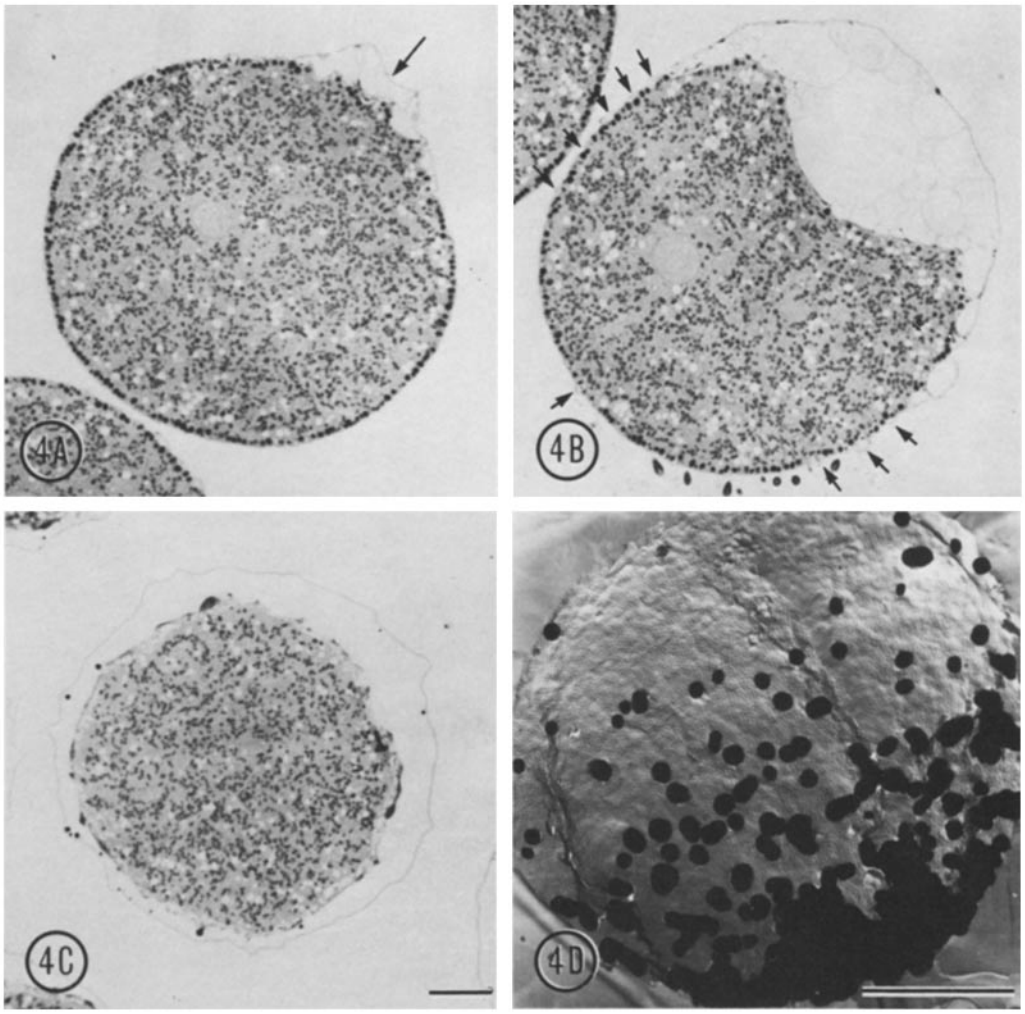

FIGURE 4 The wave of cortical exocytosis as seen in light micrographs of thick sections (*A*, *B*, and *C*) and in freeze-fracture replicas (*D*). (*A*) Initiation of exocytosis at the point of sperm attachment, as indicated by localized elevation of the vitelline layer (arrow). (*B*) Propagation of the wave. At the wave "front" there are numerous small elevations of the vitelline layer (arrows) which in electron micrographs correspond to sites where single granules have been released. (*C*) Exocytosis is complete as indicated by a fully elevated fertilization membrane and absence of cortical granules. (*D*) "Face-on" view of the exocytotic wave in a freeze-fracture replica. Sites where granule membranes have fused with the plasma membrane are blackened out to illustrate the gradient of exocytosis. Eggs were fixed at 30-s postinsemination (*A* and *B*) or at 60 seconds (*C* and *D*). Bar, 10 μm. *A*, *B*, and *C*, × 800. *D*, × 2,000.

and confluent at the back of the wave (Fig. 6). Remarkably, many patches of low particle density could be found on these flat areas, suggesting that granule membrane does not mix with the plasma membrane as fast as the granule can collapse.

All of these structural features of the exocytotic wave looked the same in aldehyde-fixed eggs as in quick-frozen eggs. The one striking difference was that in aldehyde-fixed and glycerinated eggs it was common to find small groups of exocytotic stomata within the domain of a single cortical granule (Fig. 7*A*). In fortunate fractures it was possible to confirm that these represented multiple fusion points between one granule membrane and the plasma membrane (Fig. 7*B*). Some of these spots of fusion contained etchable material in their cen-

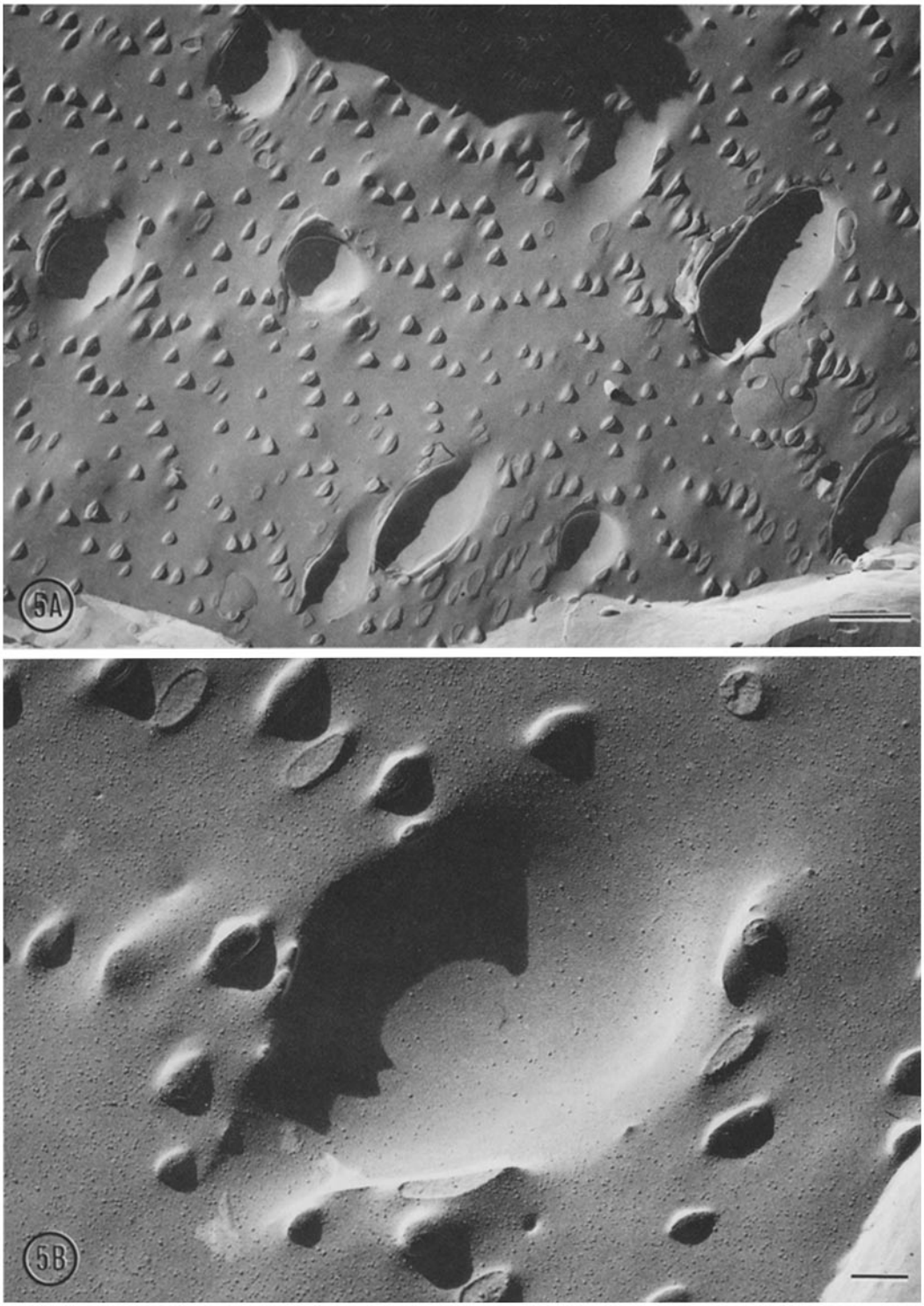

FIGURE 5 (*A*) The "front" of the exocytotic wave. Depressions resulting from the fusion of single cortical granules with plasma membrane are well separated by unchanged plasma membrane. The specimen was quick-frozen 30 s postinsemination. Bar, 1 μm. × 12,000. (*B*) A site where granule and plasma membranes have fused. The difference in particle density between these membranes suggests that some of their components have not yet mixed. Specimen was quick-frozen 30 s postinsemination. Bar, 0.2 μm. × 42,000.

FIGURE 6 Late in exocytotic wave. The egg surface is composed almost entirely of fused granule membranes in various stages of collapse. Small clumps and single rows of the original microvilli still remain. The specimen was quick-frozen 30-s postinsemination. Bar, 1 μm. × 12,000.

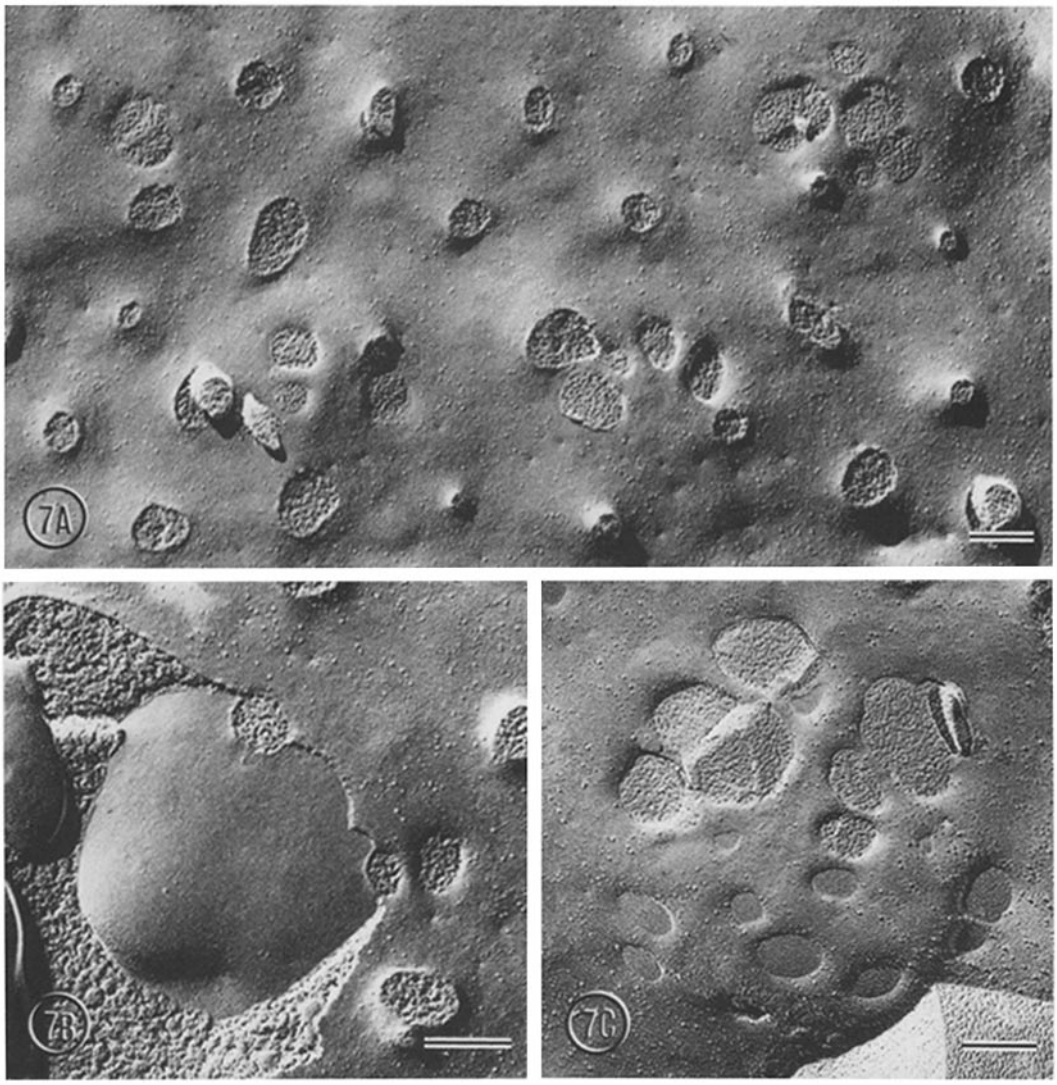

FIGURE 7 (*A*) Groups of etchable pores seen during cortical granule exocytosis in eggs that were fixed with glutaraldehyde, glycerinated (30% glycerol) and quick-frozen. Bar, 0.2 μm. × 40,000. (*B*) A fracture that jumped from plasma to granule membrane shows that the pores represent spots where these have fused. Specimen was fixed, glycerinated (20% glycerol) and quick-frozen. Bar, 0.2 μm. × 55,000. (*C*) Some pores contained a diaphragm of intramembrane particle-free membrane that was not etchable. Specimen was fixed, glycerinated (30% glycerol) and quick-frozen. Bar, 0.2 μm. × 50,000.

ters, indicating that they were completely open pores into the underlying granule lumens; others had smooth, flat, non-etchable centers which indicated that some portion of the two intervening membranes still separated the granule lumen from the outside (Fig. 7 *C*). Such remaining "diaphragms" were free of intramembrane particles and appeared in many instances to fracture as if they were composed of a single membrane bilayer which had become continuous with both granule and plasma membranes. Identical diaphragms have been seen in chemically fixed zoospores (29) and thought to be intermediates in membrane fusion. Chemically fixed eggs also displayed smaller pinpoint contacts between granule and plasma membrane, which could be seen as depressions in the plasma membrane (large arrow, Fig. 8) and as corresponding bumps in the granule

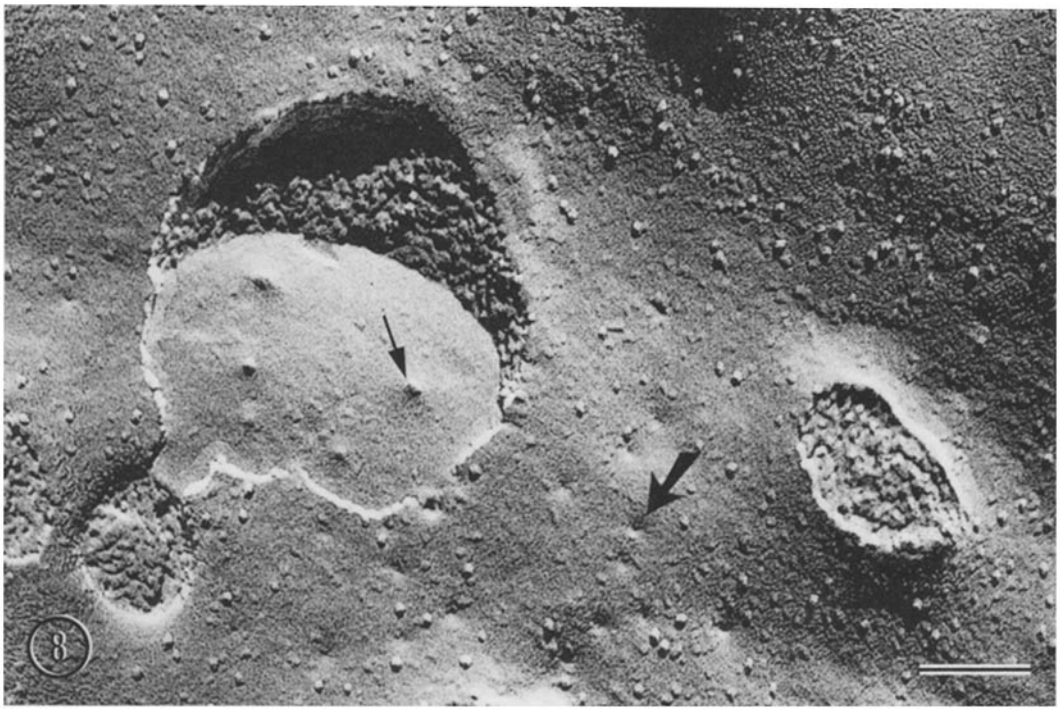

FIGURE 8 Plasma membrane dimples (large arrow) and corresponding bumps (small arrow) on the granule membrane whose contact may represent initial stages of glycerol-induced fusion. Specimen was fixed, glycerinated (30% glycerol) and quick-frozen. Bar, 0.1 μm. × 140,000.

membrane (small arrow, Fig. 8).

In contrast to these observations on chemically fixed eggs, quick-frozen eggs never displayed such multiple foci of fusion and thus we began to suspect that these structures were produced either by the aldehyde fixation of eggs or by the glycerination of fixed eggs. We found that they were not a result of fixation alone, because aldehyde-fixed eggs looked just like unfixed eggs when they were quick-frozen without glycerination. On the other hand, the putative "fusion intermediates" were easy to find on eggs that had been glycerinated after fixation, and their abundance increased progressively as the glycerol concentration was increased from 10 to 20 and 30%. They appeared to be a direct consequence of the exposure to glycerol and not just a result of the slower method of freezing used for glycerinated eggs, because they were visible even when eggs were quick-frozen after glycerination. We did this experiment because slow freezing is known to produce artifactual apposition of membranes in certain situations. (In our case, however, slow freezing of fixed eggs in the absence of glycerol produced severe ice crystal damage but no such contacts.)

Further evidence that the putative "fusion intermediates" in question were artifacts resulting from exposure to glycerol was that similar interactions appeared between adjacent granule membranes, even in unfertilized eggs where the granules had not yet become involved in exocytosis. Here, too, multiple foci of fusion could be found between single pairs of granules, and some of these foci were totally open (Fig. 9*A*) while others were bisected by unetchable, particle-free "bilayer diaphragms" (Fig. 9*B*). In fact, adjacent granule membranes appeared more sensitive to this glycerol effect than did the plasma membranes, since they were often fused by 10% glycerol while granule:plasma membrane fusions became abundant only in 20 and 30% glycerol.

Exocytosis of all cortical granules probably adds enough membrane to the plasma membrane to double its surface area (34) which may have accounted for the scalloped appearance of the discharged eggs that we prepared for thin sections

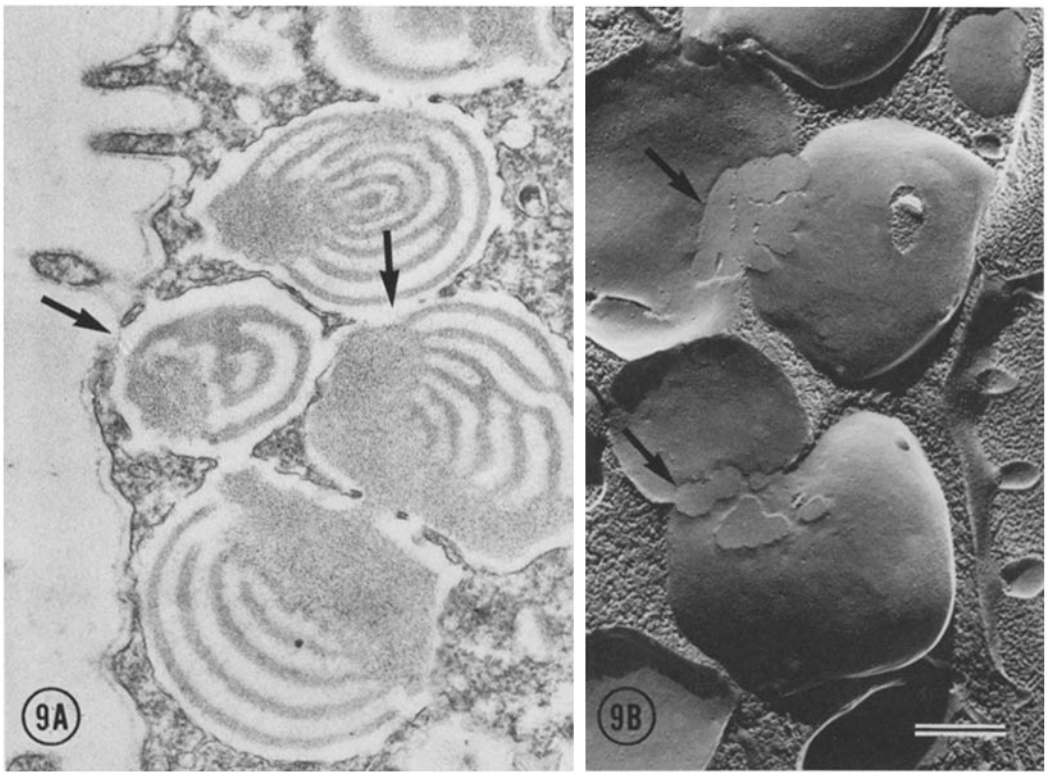

FIGURE 9 (*A*) Electron micrograph of glycerol-induced fusion between plasma and granule membranes (arrows) and between membranes of adjacent granules. Specimen was fixed, passed through 30% glycerol in seawater for 1.5 h, postfixed in OsO_4, then embedded. (*B*) Freeze-fracture replica showing glycerol-induced fusion between adjacent cortical granules. Fusion sites often contained a non-etchable, particle-free membrane diaphragm that was shared by both granule membranes (arrows). Specimen was fixed, glycerinated (10% glycerol) and quick-frozen. Bar, 0.4 μm. × 30,000.

(Fig. 10*A*). The surfaces of such eggs, fixed 60 s after insemination, were comparatively free of microvilli and possessed only a few blunt protrusions. Their vitelline layers were thickened and raised ~5 μm above the surface, thus constituting the fertilization membrane. The intervening space was filled with dense material and lipid vesicles. Freeze-fracture replicas of such discharged eggs also demonstrated the redundancy in their surface membrane and the paucity of microvilli (Fig. 10*B*), and displayed the peculiar lipid vesicles trapped under the fertilization membrane.

Eggs fixed 2 min after insemination displayed newly formed microvilli that were highly arborized (Fig. 11*A* and *B*). These microvilli looked to us like quite new structures, not merely elongations of the preexisting microvilli as has been previously stated (7). Thin sections revealed that coated pits were abundant in the plasma membrane of eggs fixed at this time (arrows, Fig. 11*A*, and insert) and freeze-fracture displayed the characteristic deployment of coated pits around the broad bases of the new microvilli (Fig. 12). Higher magnifications of the freeze-fracture views of these pits illustrated that the patch of plasma membrane involved possessed a relatively high concentration of large intramembrane particles (Fig. 12, *inset*).

In eggs fixed 5 min after insemination, the microvilli appeared more elongated still, and their processes often anastomosed to produce cavities or tunnels in the cell surface (Fig. 13*A*). Freeze-fracture replicas showed that the bases of these microvilli had become connected to form ridges which crisscrossed to form a network on the cell surface (Fig. 13*B*). This network has been seen before in scanning electron micrographs of fertil-

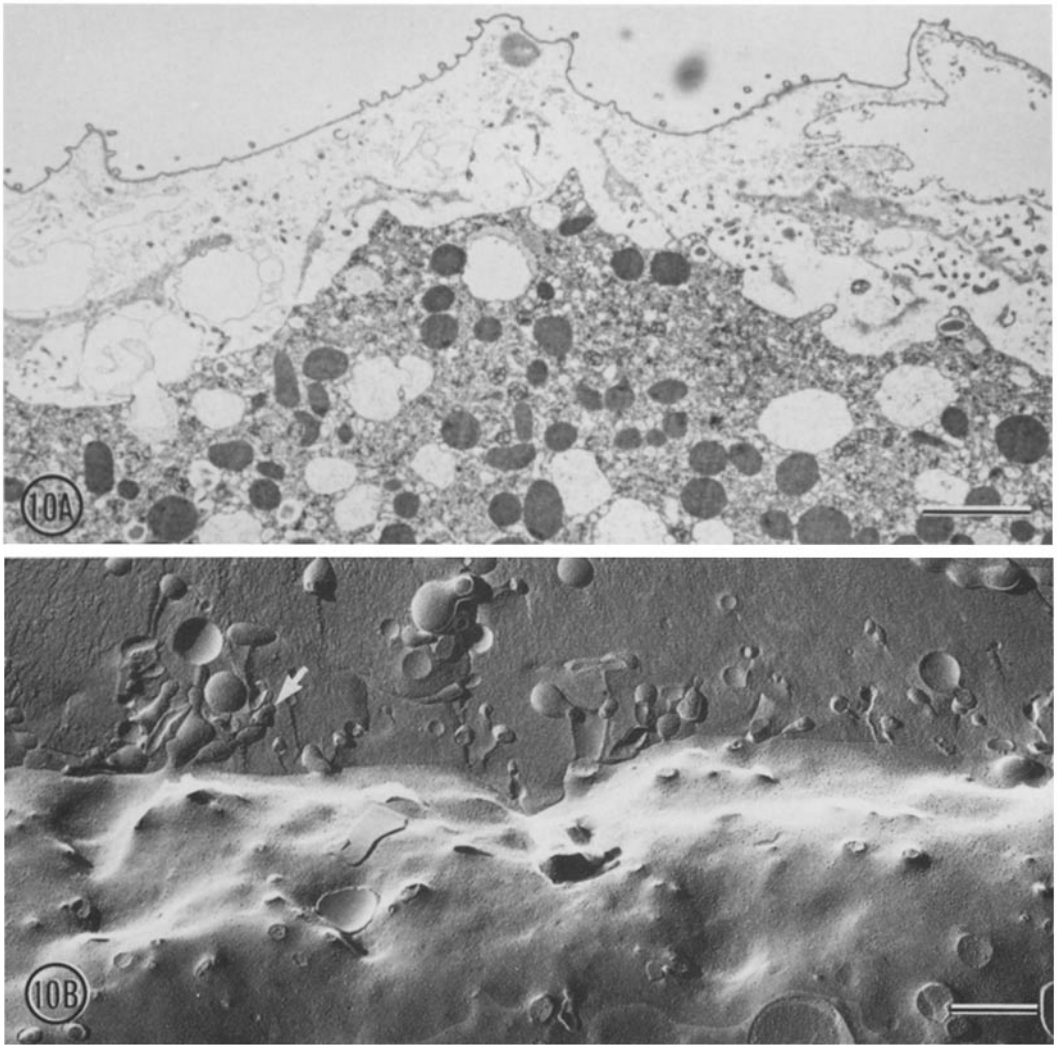

FIGURE 10 (*A*) Electron micrograph of the egg surface at 1 min postinsemination. Exocytosis is complete and the fertilization space is filled with vesicles and dense secretory products. The cell surface is scalloped, a redundancy probably due to the rapid addition of granule membrane. Bar, 2 μm. × 7,000. (*B*) Freeze-fracture replica of the egg plasma membrane at 1 min postinsemination. The surface is redundant with only a few scattered microvilli. However, these microvilli (arrow) are unlike the short, stubby ones seen in unfertilized eggs; their length forecasts the rapid growth of microvilli that will occur in the next few minutes. Specimen was fixed, glycerinated (30% glycerol) and frozen in Freon. Bar, 0.5 μm. × 22,000.

ized eggs (7). The plasma membrane between the ridges was relatively smooth and displayed only a few dimples that could have been coated pits.

DISCUSSION

In this freeze-fracture study of sea urchin eggs, we were unable to catch intermediates in membrane fusion by quick freezing, but instead found that certain structures thought to be intermediates could be found only on eggs exposed to glycerol after fixation. These structures included small clusters of fusion spots between the cortical granule and plasma membranes or between adjacent granule membranes. Some of these fusion spots appeared to be single bilayer diaphragms. Fixation alone did not produce these structures, but they appeared to be a result of exposure to high concentrations of glycerol. Glycerol at these concentrations is a dehydrant and has been used to make living cells fuse (2); but, in the present situation, the fusion observed occurred between fixed membranes, so its intermediate stages were not necessarily relevant to the way in which exocytosis occurs normally.

That glutaraldehyde-fixed membranes could be made to fuse by glycerol should not have been surprising, in view of recent studies which show that the membrane bilayer remains in a very labile state during and after fixation. Hasty and Hay (11) have shown that aldehyde-fixed fibroblasts develop blisters in the plasma membrane which are free of IMPs and are often puffed up from beneath by closely-apposed bilayer vesicles. Similar IMP-free vesicle contacts were found in cholinergic nerve terminals when they were fixed in aldehydes, especially when the fixative contained high concentrations of divalent cations (4, 12, 13). Furthermore, aldehyde fixation is known to produce vesiculation of membrane at sites of exocytosis in both sea urchin eggs (5, 7) and mast cells (19, 20). Thus, movement of IMPs and localized distortion of the membrane bilayer can occur during, or even after, fixation. Movement of IMPs during fixation has also been found to occur at gap junctions in ciliary epithelia (31). The typical close-packed hexagonal array of IMPs which characterized gap junctions was found only in epithelia that were aldehyde fixed; when they were quick-frozen, instead, gap junction particles were found to be more randomly dispersed.

Such aldehyde-induced changes are similar to what others have described as natural intermediates in membrane fusion. Membrane contacts cleared of IMPs have been described as a stage of exocytosis in mast cells (6, 20), pancreatic beta cells (24), a stage of apocrine secretion in the mammary gland (28), a stage of acrosome fusion in sperm (9), and a stage of intercellular fusion between myoblasts (17). Formation of small IMP-free vesicles has been described as a step in myoblast fusion (17). In addition, the glycerol-induced changes that we saw in eggs were almost identical to the IMP-free single bilayer diaphragms that have been described as intermediates of exocytosis in zoospores (29). These similarities suggest that fixation and glycerination could play a role in determining the abundance or final appearance of any of these structures.

One could point out that most of these structures, including the bilayer diaphragms seen in zoospores, have been found in both thin sections and freeze-fracture. Indeed, the multiple openings into cortical granules that we saw in glycerinated and freeze-fractured eggs were equivalent to what Millonig (22) observed in thin sections of sea urchin eggs. Although this could argue for validity of these structures, it should be appreciated that both methods of tissue preparation involve dehydration of membranes that may not have been completely stabilized by fixation, and may be subject to further changes.

Even though some of the membrane interactions just mentioned could be artifacts, we should stress that they have usually been found only in the regions where, and at the times when, membranes are naturally fusing. Thus, they may herald underlying changes. For example, in the case of cortical granule exocytosis, the ability of glycerol to induce multiple sites of fusion between granule and plasma membranes may indicate that the plasma membrane becomes susceptible to fusion over quite a broad area during exocytosis. This might happen if, for example, it were to lose a cytoplasmic coat which normally blocked membrane contact.

Quick freezing can be thought of as sampling one particular amount in a biological process, because the interval between the time when it starts to affect the process and the time when it completely halts it is relatively brief. Elsewhere we demonstrate that it is ~2 ms or less (14). Thus, when a process is going on repetitively or continuously, as is cortical granule exocytosis at the peak of the wave, quick freezing should yield images of each stage in the process, and in a relative abundance that should be proportional to how long

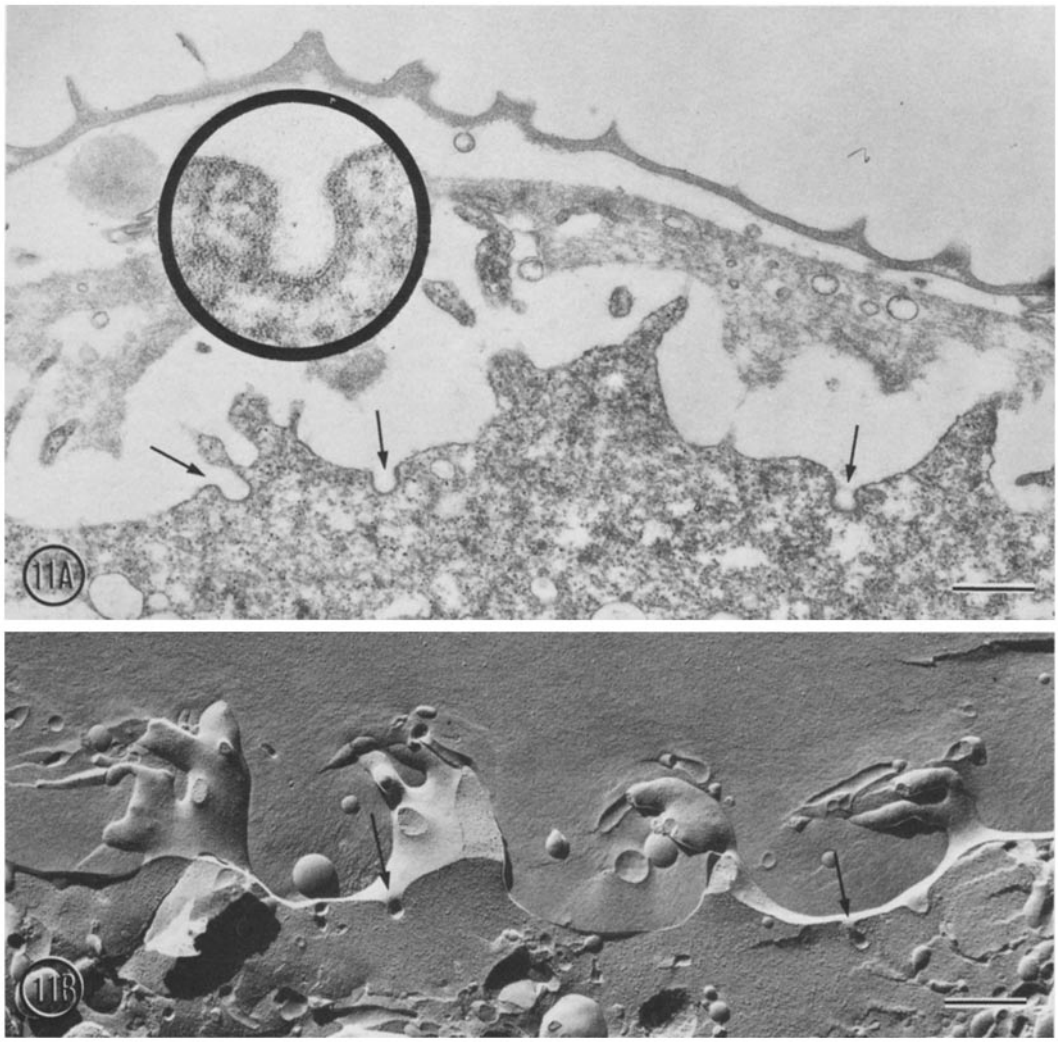

FIGURE 11 (*A*) Electron micrograph of the egg periphery at 2 min postinsemination showing highly arborized microvilli. Between the microvilli are numerous invaginations (arrows) which have dense coats typical of endocytosis (*inset*). Bar, 0.5 μm. × 23,000; *inset*, × 120,000. (*B*) Crossfracture of the egg periphery 2 min after insemination. Numerous, long, and highly branched microvilli have appeared. Specimen was fixed, glycerinated (30% glycerol) and frozen in Freon. Bar, 0.5 μm. × 22,000.

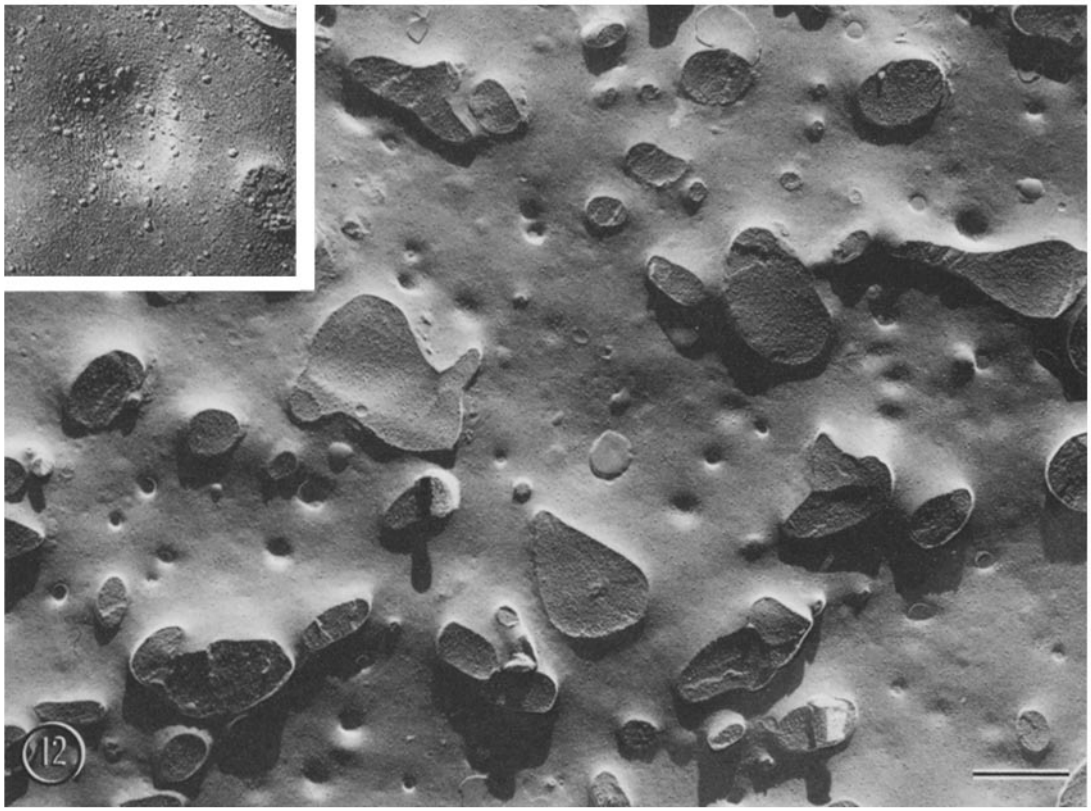

FIGURE 12 Freeze-fracture replica of the egg plasma membrane 2 min after insemination (P face). Coated pits are interspersed among the large, mesa-like bases of crossfractured microvilli and, as seen in the inset, are associated with large intramembrane particles. Bar, 0.5 μm. × 25,000; *inset*, × 80,000.

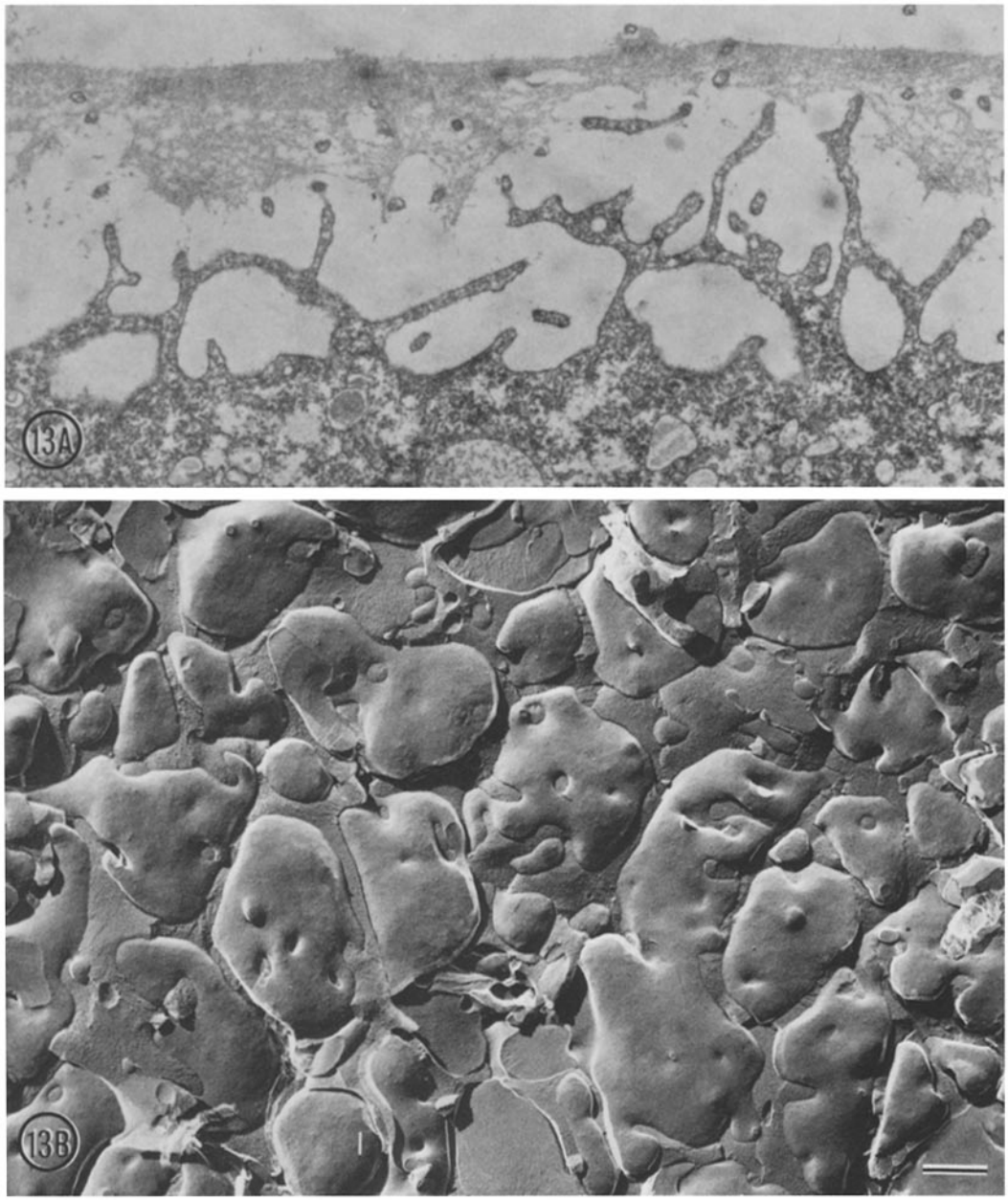

FIGURE 13 (*A*) Electron micrograph of the egg periphery 5 min postinsemination. Microvilli now have long thin branches which occasionally join to produce tunnels or cavities in the extracellular space. (*B*) Freeze-fracture replica of the egg plasma membrane 5 min postinsemination. This E-face view shows that the microvilli (which are all cross-fractured) have become arranged in a network of ridges, with clear plasma membrane between. Bar, 0.5 μm. × 18,000.

each stage lasts. There should be little worry that certain intermediates in the process will accumulate because later stages are blocked preferentially, which can be a problem with aldehyde fixation (15). Thus, collapse of granule membranes after exocytosis must require several seconds because in every case this process was caught in a variety of stages just after the passage of the wave front. Its duration is 3–5 s if one assumes a wave velocity of ~5 μm/s. In comparison, the initial stages of exocytosis must be extremely fast, because we saw over 1,000 granule openings without finding anything that looked like a preliminary stage. Unfortunately, if such stages lasted, say, only 5 ms of the overall three to 5 s, they would comprise only one out of 500 of the openings we saw and could have been overlooked. If their lifetime were shorter, they would be even more rare.

The later consequences of exocytosis were faithfully portrayed by either chemical fixation or quick freezing, indicating that they occur relatively slowly. Although collapse of the granule membrane after exocytosis took several seconds, mixing of some granule and plasma membrane components, as judged by IMP distribution, took longer. The plasma membrane redundancy created by exocytosis appeared to be resolved by the formation of coated pits, which became plentiful by 2 min after fertilization and died out again after 5 min. Tracer experiments are needed to confirm that these coated pits were indeed endocytotic and that they occur in sufficient quantity to reduce the surface area. The excess surface area was also used in formation of new microvilli, highly anastomotic in form, within 5 min after fertilization. The relationship of these shape changes to reorganization of filimentous structure in the cortical cytoplasm of eggs in a subject of current inquiry.

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