COMPARISON OF QUICK-FROZEN AND CHEMICALLY FIXED SEA-URCHIN EGGS: STRUCTURAL EVIDENCE THAT CORTICAL GRANULE EXOCYTOSIS IS PRECEDED BY A LOCAL INCREASE IN MEMBRANE MOBILITY

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SUMMARY
Eggs of the purple sea-urchin, Strongylocentrotus purpuratus, were fertilized and fixed with 2% glutaraldehyde at various stages during cortical granule exocytosis. Fixation resulted in membrane blebs being formed precisely at the point of incipient granule fusion. These blebs pinched off to form the membranous vesicles frequently seen in exocytic pockets and in the perivitelline space. In contrast, eggs that were fixed with osmium tetroxide or were quick-frozen without chemical fixation, showed no signs of bleb or vesicle formation. Rather, fusion of each granule appeared to begin at a single minute pore, 30–50 nm in diameter, which then enlarged. We suggest that formation of blebs during glutaraldehyde fixation is an artifact that is caused by a highly localized and transient increase in membrane mobility. Normally, this increased mobility facilitates fusion of granule and plasma membranes, but in the presence of glutaraldehyde it leads to large-scale distortions of these fusing membranes.

INTRODUCTION
During exocytosis, fusion of the secretory granule membrane with the plasma membrane occurs within a few milliseconds (Douglas, 1974; Heuser et al. 1979). This period is extremely short in comparison with the time needed to fix a cell preparation chemically for electron-microscopic examination. For example, glutaraldehyde, the most common primary fixative, probably requires a period of at least a few seconds to halt cell processes. Since fixation is slow in comparison with the process being preserved it is not surprising that fusion events in partly fixed membranes might lead to unnatural or exaggerated structures, that is, artifacts.

We have previously documented the presence of artifacts in sea-urchin eggs undergoing cortical granule exocytosis. After the egg is fertilized, exocytosis begins at the point of sperm entry and spreads as a wave over the entire egg surface, reaching the opposite pole about 30 s later (Eddy & Shapiro, 1976; Chandler & Heuser, 1979). Conveniently, what is a temporal sequence of events in most secretory cells is laid out as a spatial sequence of events on the surface of the egg. The cortical granules at the

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front of the wave appear to be undergoing the initial steps of fusion with the plasma membrane. In glutaraldehyde-fixed eggs, this region is dotted with groups of multiple pores leading into individual granules, and some pores are covered with intramembrane particle (IMP)-free diaphragms that are bilayers continuous with both plasma and granule membranes. Further study has shown both pores and IMP-free diaphragms to be artifacts created by glycerination following fixation (Chandler, 1979; Chandler & Heuser, 1979).

In this study we demonstrate the presence of a second fixation artifact created by glutaraldehyde treatment during cortical granule exocytosis—the formation of large membrane vesicles at the initial site of fusion between plasma and granule membranes. In comparison, when exocytosis is halted by a non-chemical method such as quick-freezing, there is no vesicle formation. Fusion appears to begin with a single small opening that quickly enlarges. This comparison warns us that glutaraldehyde causes artifacts that can be mistaken for structures reported to be intermediate stages of exocytosis. However, glutaraldehyde-induced vesiculation of the plasma membrane also suggests that there is a highly localized increase in distendability of the plasma and secretory granule membranes just before fusion. This period of distendability appears to be lengthened in the presence of glutaraldehyde, leading to movement of membrane into the site of fusion and its ballooning outward into the extracellular space.

MATERIALS AND METHODS

Pacific sea-urchins, Strongylocentrotus purpuratus, were obtained commercially and maintained at 11°C in artificial sea water (‘Instant Ocean’, Aquarium Systems, Inc. Eastlake, Ohio). Shedding of gametes was induced by injecting 0.5 M-KCl into the body cavity. Eggs collected in sea water were dejellied by three passages through a 150 μm mesh nylon cloth, then resuspended in fresh sea water and kept at 10°C until use. Sperm was collected ‘dry’, kept at 5°C, and 10 min before fertilization, diluted 1:100 with sea water. Gametes were brought to room temperature for all experiments.

Fertilization was carried out by mixing 1 ml of a 1% (v/v) sperm suspension with 6 ml of a 3% (v/v) egg suspension. At appropriate times, fertilized eggs were fixed by adding an equal volume of 4.0% glutaraldehyde in diluted sea water (80% of normal tonicity). Fixation was continued for 1 h at room temperature, and the eggs were then washed in sea water.

For freeze-fracture, eggs were next suspended in 30% glycerol/70% sea water (v/v) for 1.5 h, packed by centrifugation (100 g, 1 min), a drop of packed cells was sandwiched between two gold-alloy specimen carriers and frozen in melting Freon 22. These samples were then fractured by the double replica method (Wehrli, Mühlethaler & Moor, 1970) in a Balzers 400 freeze-etch unit (Balzers AG, Balzers, Lichtenstein), and replicated with platinum–carbon from an electron beam gun mounted at 45°. Specimens were either fractured at −130°C without etching or fractured at −110°C and allowed to etch for 20 s before replication. Replicas were cleaned and viewed in a Philips 300 electron microscope at 80 kV.

Other eggs were post-fixed in a 1% (w/v) osmium tetroxide in diluted sea water (75%) for 1 h at room temperature, washed in sea water, and block-stained with 1% (w/v) uranyl acetate in 50 mM-sodium acetate buffer (pH 5.0), for 1 h in the dark at room temperature. After the cells were dehydrated in a graded series of ethanol and embedded in Araldite, silver sections were cut, and stained with 10% uranyl acetate in 50% methanol (w/v), followed by 0.4% lead citrate in 0.15 M-NaOH. As an alternative, some samples were fixed directly with 2% osmium tetroxide in sea water for 1.5 h at room temperature, washed with sea water, then block-stained and processed for thin sectioning as described above.

Quick-freezing was done with a machine of the type designed by Heuser et al. (1979) utilizing
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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 custom-cut coverglasses that had been coated with protamine sulphate as previously described (Chandler & Heuser, 1979). Eggs were allowed to settle on the coverglasses, then fertilized by dipping into a 1% (v/v) sperm suspension, and excess eggs were then washed away. The coverglass, with adherent eggs, was placed at the tip of the machine plunger and dropped onto the cold block. Subsequent freeze-fracture and replication of quick-frozen samples was carried out as described by Heuser et al. (1979).

Quick-frozen specimens to be freeze-substituted were placed on frozen acetone containing 4% osmium tetroxide and covered with liquid nitrogen. The acetone was allowed to thaw and the specimen, now immersed in the acetone–osmium tetroxide mixture, was kept at −70°C for 48 h, then at −20°C for 2 h. The sample was then washed in cold ethanol, block-stained with 1% uranyl acetate in ethanol for 1 h in the dark at room temperature, and embedded in Araldite.

Specimens to be deeply etched (as in Fig. 5) were first fixed in 2% glutaraldehyde in 80% sea water, washed with water, and quick-frozen. The frozen specimen was then fractured in a Balzer’s unit, etched for 3 min at −100°C and rotary-replicated with platinum–carbon at an angle of 25°.

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

In eggs of *S. purpuratus*, cortical granule exocytosis begins approximately 20 s after insemination and sweeps as a wave over the egg surface during the subsequent 30 s. At the front of the wave only a few single granules fuse to form pockets. In glutaraldehyde-fixed cells these pockets are frequently filled with numerous vesicles as shown in Fig. 1. At higher magnification the vesicles are seen to consist of membranes of normal bilayer structure (data not shown). In freeze-fracture replicas, they appear spherical, some vesicles being smooth while others have numerous intramembrane particles (Fig. 2).

These vesicles appear to form at sites where granule and plasma membranes are about to fuse. Fig. 3 shows that within a narrow region where these two membranes are in closest contact (arrows), the plasma membrane has expanded to form a bleb that extends into the extracellular space. The granule membrane also appears deformed in this area although to a lesser extent. Further expansion of the plasma membrane forms larger rounded blebs (see Fig. 4). These, by pinching off, could give rise to the vesicles seen in the exocytic pockets as well as in the perivitelline space after passage of the exocytic wave. These vesicles range from 0.5 μm to as large as 5 μm in diameter, and in some cases are multiwalled.

Three observations lead us to believe that vesicle formation occurs only when exocytosis takes place in the presence of glutaraldehyde. First, vesicles are not seen when unfertilized eggs are fixed nor are they seen in eggs fixed after cortical granule exocytosis is complete. Second, eggs that have been fixed in glutaraldehyde, then quick-frozen, freeze-fractured and deeply etched, still show evidence of this artifact (see Fig. 5). Large membrane blebs are seen extending from cortical granules into the perivitelline space, with some blebs containing filamentous material that appears to have been discharged from the granule. The cortical granule interior itself contains a fibrous superstructure arranged in layers (Fig. 5), which correspond to the characteristic striations seen in thin sections (see Fig. 1). Because quick-freezing
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and freeze-fracture bypass the tissue-dehydration steps needed in conventional freeze-fracture or thin-section preparation, it is clear that blebs and vesicles must form during fixation itself, rather than during subsequent steps in tissue processing.

Similar results are obtained in specimens that have been fixed with glutaraldehyde, quick-frozen, then freeze-substituted and fixed with osmium tetroxide at low temperature (−20°C) before embedding. Vesicles are still present at sites of exocytosis (Fig. 6). In contrast, when osmium tetroxide is used as a primary fixative instead of glutaraldehyde, membrane vesicles are not present at sites of exocytosis (see Fig. 7) or within the perivitelline space after exocytosis. This is the third observation indicating

Fig. 1. An electron micrograph of an S. purpuratus egg undergoing cortical granule exocytosis. An arrow points to membrane vesicles often seen in exocytic pockets. The specimen was fixed with 2% glutaraldehyde at 30s post-insemination (p.i.). ×35 000.

Fig. 2. A freeze-fracture replica of the egg plasma membrane (P-face). Two spherical membrane vesicles lie within a crossfractured exocytic pocket. On the E-face of these vesicles are numerous intramembrane particles (IMPs), which in some areas are grouped into clusters. The specimen was fixed in 2% glutaraldehyde at 20 s p.i. ×35 000.

Fig. 3. In glutaraldehyde-treated eggs, the beginning of exocytosis is heralded by expansion of the plasma membrane at a small region of interaction between granule and plasma membranes (between arrows). The specimen was fixed at 30 s p.i. ×100 000.

Fig. 4. A large, spherical bleb, continuous with the plasma membrane (pm), has formed at the site of exocytosis. Such blebs appear to pinch off to form the vesicles seen in exocytic pockets. The bilayer draped over this bleb is part of such a vesicle. The specimen was fixed with 2% glutaraldehyde at 30 s p.i. ×80 000.

Fig. 5. Freeze-fracture replica showing a plasma membrane bleb filled with fibrilar material released from the interior of the cortical granule. The specimen was fixed in 2% glutaraldehyde at 30 s p.i., passed through water, quick-frozen, freeze-fractured and deeply etched. ×20 000.

Fig. 6. Membrane vesicles seen in specimens that have been fixed with glutaraldehyde at 30 s p.i., then quick-frozen and freeze-substituted. ×33 000.

Fig. 7. Exocytic pockets show no evidence of vesicle or bleb formation in specimens fixed in 2% osmium tetroxide at room temperature. ×30 000.

Fig. 8. Cortical granule exocytosis in eggs quick-frozen at 60 s p.i., then freeze-substituted and fixed with 4% osmium tetroxide at −20°C. As the granule opens, the thin collar of cytoplasm surrounding it appears to pinch off (large arrows) and form the small vesicles seen in the extracellular space (small arrows). ×50 000.

Fig. 9. Freeze-fracture replica of a quick-frozen sea-urchin egg. In unfertilized eggs many cortical granules are 'docked' at the plasma membrane and separated from it by a thin layer of cytoplasm only (arrow). ×56 000.

Figs 10—13. Cortical granule exocytosis in sea-urchin eggs, quick-frozen 20 s or 30 s post-insemination.

Fig. 10. A small pore on the P-face of the egg plasma membrane (arrow) is the earliest sign of fusion between cortical granule and plasma membranes. ×100 000.

Fig. 11. As the pore enlarges there is no indication of IMP-clearing or of bleb formation. ×100 000.

Fig. 12. Expansion of the pore produces a wide opening into the interior of the granule. ×75 000.

Fig. 13. Finally, a large exocytic pocket is formed. The cortical granule membrane within the pocket can still be distinguished from plasma membrane by its low density of large IMPs. Fusion of a second granule is marked by a cross-fractured pore (arrow). ×34 000.
Figs 6–8. For legend see p. 27.
Figs 9–13. For legend see p. 27.
that glutaraldehyde fixation is the point at which fusing membranes form vesicles.

Quick-freezing of live eggs, followed either by freeze-substitution and embedding or by freeze-fracture has allowed us to circumvent glutaraldehyde fixation completely and view early events in cortical granule fusion unhindered by the artifacts described above. In thin sections of freeze-substituted eggs large vesicles are absent (Fig. 8). What is seen, as the exocytic pocket enlarges, is a population of much smaller vesicles about 50 nm in diameter (small arrows, Fig. 8). These may have arisen from the thin layer of cytoplasm that forms a collar around the pore leading into the pocket; the cytoplasm at each lip of this pore is constricted, as if a vesicle were about to pinch off (large arrows, Fig. 8).

In freeze-fracture replicas of quick-frozen, unfertilized eggs, cortical granules lie in a single layer immediately below the plasma membrane (Fig. 9). In some cases the granule membrane is separated from the plasma membrane by a narrow layer of cytoplasm only (arrow, Fig. 9). In freeze-substituted eggs this space between the two membranes is often filled with a dense osmophilic material that may help anchor the granule to the plasma membrane (Chandler, unpublished observations). It is in this region that the two membranes initially fuse.

Early events in exocytosis can be seen in freeze-fracture replicas of eggs quick-frozen 20–30 s after insemination. The earliest is an indentation in the plasma membrane P face, 30–50 nm in diameter, that could represent the initial contact or fusion of this membrane with the underlying granule membrane (Fig. 10). Next, somewhat larger, single pores, 0.1–0.2 μm in diameter are seen leading into granules (Fig. 11); these pores appear to enlarge to produce openings of larger diameter (Fig. 12), until finally a shallow pocket is formed (Fig. 13). The membrane within the pocket is characterized by a low density of large IMPs similar to those seen on the P-face of cortical granule membranes before fusion.

DISCUSSION

Glutaraldehyde is a common primary fixative used in preparing tissues for embedding and for freeze-fracture electron microscopy. Its properties as a fixative come from the fact that it is a bifunctional aldehyde that forms adducts with amino groups, thereby crosslinking proteins (Molin, Nygren & Dolonius, 1978; Bullock, 1984). In comparison, it is a relatively poor fixative for membranes since it does not react readily with the phospholipids commonly present in biological membranes (Holt & Hicks, 1965; Cope & Williams, 1969). This differential ability to fix proteins but not lipids may account for the fact that glutaraldehyde fixation results in a number of artifacts in membranes that are undergoing rapid changes in structure.

Fusion of secretory granule and plasma membranes during exocytosis represents an
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important example. The literature provides evidence of at least three distinct fixation
artifacts that can occur during this process. The first artifact is the common observa-
tion that, before exocytosis, the plasma membrane overlying the secretory granule
becomes cleared of intramembrane particles (IMPs). IMP clearing is seen during
exocytosis in mast cells (Lawson et al. 1977), sperm (Friend, Orci, Perrelet &
Yanagimachi, 1977), islet cells (Orci, Perrelet & Friend, 1978), the parotid and
lacrimal glands (Tanaka, De Camilli & Meldolesi, 1980) and in a host of other secre-
tory tissues (Amherdt, Baggliolini, Perrelet & Orci, 1978; Swift & Mukherjee, 1978;
Theodosis, Dreifuss & Orci, 1978), as well as during myoblast fusion (Kalderon &
Gilula, 1979) and polyethylene glycol-induced fusion of erythrocytes (Knutton,
1979). This has been interpreted to mean that fusion actually takes place in lipid-rich
domains that have been cleared of membrane proteins. Such IMP clearings, however,
are not seen when exocytosis is arrested by quick-freezing rather than chemical fixa-
tion. Quick-freezing by Heuser's method (Heuser et al. 1979) halts biological action
within a few milliseconds and thus should be able to preserve membrane-fusion events
lasting only a few milliseconds better than does chemical fixation. Thus, the fact that
no sign of IMP clearing is seen in quick-frozen mast cells (Chandler & Heuser, 1980),
amoebocytes (Ornberg & Reese, 1981) or sea-urchin eggs (this study) even at the
earliest stages of granule fusion, whereas it is commonly seen in glutaraldehyde-fixed
cells, leads us to conclude it is an artifact.

A second artifact is the fusion of membranes after fixation. In glutaraldehyde-
treated sea-urchin eggs, one can find examples at the onset of cortical granule
exocytosis in which multiple aqueous pores join a single granule with the extracellular
space (Chandler & Heuser, 1979). Some of these pores are covered by a single IMP-
free bilayer that is continuous with both plasma and granule membranes (Chandler,
1979). Further study showed that both pores and IMP-free bilayers were formed
during glycerol dehydration after fixation rather than before or during fixation. This
means that the IMP-free bilayer, claimed to be an intermediate state in membrane
fusion in several studies (Pinto da Silva & Nogueira, 1977; Kalderon & Gilula, 1979),
could simply reflect the inability of glutaraldehyde to prevent glycerol-induced mem-
brane fusion.

A third artifact resulting from glutaraldehyde fixation is documented in this study.
At the initial site of fusion between plasma and granule membranes, the plasma
membrane appears to expand and to balloon outwards (Figs 3–5). This bleb then
appears to pinch off, giving rise to the many vesicles seen within exocytic pockets of
glutaraldehyde-fixed eggs (Fig. 1). Similar blebs and vesicles have been seen in
glutaraldehyde-treated mast cells (Lagunoff, 1973; Lawson et al. 1977), parotid and
lacrimal glands (Tanaka et al. 1980), and in myoblasts (Kalderon & Gilula, 1979),
nearly always at a site of membrane fusion. Such artifacts can be observed even in cells
where membrane fusion is not an obvious activity (Hasty & Hay, 1978; Shelton &
Mowczko, 1978).

Each of the three artifacts described above could be explained by the inability of
glutaraldehyde to fix lipids. For example, glycerol and its derivatives are known to be
fusogenic (Quirk et al. 1978; Knutton, 1979; Aldwinckle et al. 1982) and are capable
of causing IMP redistribution in membranes of live cells (McIntyre, Gilula & Karnovsky, 1974). Thus, the fact that glycerination, after fixation, causes membrane fusion and IMP clearing is surprising only because it demonstrates that even long-term exposure to glutaraldehyde (2% for 1 h) is insufficient to prevent movement of IMP and phospholipid. This conclusion is strengthened by studies showing that membrane fluidity as measured by electron spin resonance is reduced to near zero by osmium tetroxide but is relatively unaffected by glutaraldehyde (Jost, Brooks & Griffith, 1973).

The failure of glutaraldehyde to halt lipid mobility while at the same time crosslinking proteins may be responsible for the formation of blebs and vesicles during the fixation process. It seems reasonable to assert that these artifacts occur when exocytosis is attempted in a cell that is already partially fixed. This could mean, for example, that cytoskeletal proteins in the cytoplasm have been fixed in place, and that the exocytic pocket, now set in 'cement', cannot open up properly, or that granule contents are fixed and their normal hydration, swelling and expulsion is prevented. At the same time, however, mechanisms that normally increase the fusibility of these bilayers just before exocytosis may be free to act for abnormally long periods, thus producing excessive mobility and deformability of these membranes just at the point where they are about to fuse. Formation of blebs and vesicles, therefore, may be an exaggeration of a physiological process, which under normal cellular control occurs only transiently and only in a very highly localized area so as to facilitate the initial fusion event.

What this local increase in deformability represents is a matter for discussion. First, it is clear that formation of a bleb at the site of granule fusion requires a large increase in membrane surface area. For example, if one compares the surface area of the spherical bleb in Fig. 4 (3.0 \( \mu \text{m}^2 \)) with the surface area of the circular neck by which it is attached to the egg (0.3 \( \mu \text{m}^2 \)), there has been a tenfold increase. Such an increase in surface area could not be accounted for by increased distance between phospholipid molecules alone. There must be bulk movement of membrane and protein into the bleb.

Second, local deformation of the bilayer suggests that either the bilayer has been altered in its physical properties or its linkage with the underlying cytoskeleton has been severed. In the former case, it is relevant that studies using artificial phospholipid bilayers have shown that calcium ions, the usual physiological trigger for exocytosis, can induce lipid phase separations in multicomponent systems (Papahadjopoulos, Post, Schaeffer & Vail, 1974). Phase separations not only lead to increased fusibility of these bilayers (Papahadjopoulos et al. 1974) but can also lead to decreased surface tension and therefore greater distendability of the bilayer (Purdon, Tinker & Neumann, 1976). In the latter case, retraction of the cytoskeleton from a small domain between the granule and plasma membranes could leave these membranes unsupported, highly distendable, and easily capable of fusion. Indeed, small regions cleared of cytoskeletal filaments have been seen between fusing membranes in mast cells (Lawson et al. 1977), and red blood cell membranes when stripped of their underlying cytoskeletal connections do become extremely susceptible to fusion
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(Elgsæter, Shotton & Branton, 1976; Allan, Thomas & Limbrick, 1982). Such prefusion movements of the cytoskeleton, if linked to movement of membrane proteins represented by IMPs, might also account for the fact that IMP clearing is seen at sites of exocytosis in glutaraldehyde-fixed cells. For example, if glutaraldehyde blocks membrane fusion faster than it halts cytoskeletal retraction, fixation would lead to unphysiologically large areas of filament removal and IMP clearing.

Regardless of the reason for a local increase in membrane mobility, ballooning outward of either the plasma or granule membrane to form a bleb must require a pressure gradient between the extracellular space and the interior of the granule. This pressure is probably created by a movement of water due to an osmotic imbalance across these membranes. Although the degree of osmotic imbalance could depend on the osmolarity of the fixative itself, the relative hyperosmolarity of our fixative should inhibit rather than promote ballooning out of these membranes. Rather, bleb formation suggests a high osmotic pressure within the granule. Indeed, it has been postulated that a rapid increase in intragranular osmotic pressure, due to either entrance of anions (Pollard, Pazoles, Creutz & Zindler, 1979) or to dissociation of granular contents, acts to promote membrane fusion and opening of the granule during exocytosis (Cohen, Zimmerberg & Finkelstein, 1980).

Fig. 14. Steps in cortical granule exocytosis. (1) Small regions in both the plasma and granule membrane (unhatched) become unstable and highly mobile. (2) Membrane fusion begins in this region with formation of a small pore. (3) The highly mobile membrane around the pore (unhatched) forms vesicles as the pore enlarges and granule contents escape. Glutaraldehyde fixation (black arrow) prevents normal fusion of these two membranes, allowing the plasma membrane, in its highly mobile state, to expand rapidly and form a bleb. *pm*, plasma membrane; *cg*, cortical granule.
In Fig. 14 (upper left panel), the small region of increased lipid mobility that we think occurs just before exocytosis is represented by the clear, unhatched areas in the plasma and granule membranes. The ability of glutaraldehyde to exaggerate these lipid movements so as to produce blebs is illustrated by the solid arrow. In contrast, what we believe to be a more physiological sequence of events is indicated in Fig. 14 by clear arrows and numbered steps: (1) A rise in intracellular free calcium (now established as the trigger for exocytosis in sea-urchin eggs (Steinhardt, Zucher & Schatten, 1977; Baker, Knight & Whitaker, 1980) turns on enzymic activities, induces phase transitions, or initiates structural changes in the cytoskeleton that result in increased bilayer mobility within a microdomain. (2) Increased mobility promotes fusion of the plasma and granule membranes resulting in a single, narrow pore. (3) This pore rapidly expands; tiny vesicles (as seen in Fig. 8) pinch off either during pore formation or expansion as a result of the increased fusibility of membranes in this region. Evidence for formation of 'microvesicles' comes not only from thin sections of quick-frozen eggs, in which small bits of cytoplasm are seen pinching off from the lip of the pore (Fig. 8), but also from freeze-fracture replicas in which small 'peaks' of membrane are seen at the edge of the exocytic pocket (fig. 5 of Chandler & Heuser, 1979). These peaks could represent sites at which membrane is being extruded to form vesicles. Similar 'microvesiculation' of the plasma membrane has been observed in red blood cells treated with the calcium ionophore A23187 so as to increase cytosolic calcium levels (Allan, Billah, Finean & Michell, 1976; Elgsaeter et al. 1976; Vos et al. 1976; Allan, Thomas & Limbrick, 1980).

Thus, on the one hand, glutaraldehyde fixation artifacts represent abnormal structures that can be easily mistaken for physiological events. Such problems can be minimized by using alternative fixation techniques such as quick-freezing or combined fixatives such as osmium–glutaraldehyde (Hirsch & Fedorko, 1968; Lawson et al. 1977; Hasty & Hay, 1978). On the other hand, glutaraldehyde represents only one of a number of chemicals that might be used as tools to block some events in exocytosis while allowing others, such as localized lipid mobility, to be visualized in their more extreme forms.

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