A FREEZE-FRACTURE STUDY OF MEMBRANE EVENTS DURING NEUROHYPOPHYSIAL SECRETION

DENNISE T. THEODOSIS, JEAN JACQUES DREIFUSS, and LELIO ORCI

From the Department of Morphology, Institute of Histology and Embryology and the Department of Physiology, University of Geneva Medical School, CH-1211 Geneva 4, Switzerland

ABSTRACT

Freeze-fracture was used to study the membrane events taking place during neurosecretory granule discharge (exocytosis) and subsequent membrane internalization (endocytosis) in axons of neurohypophyses from control and water-deprived rats.

En face views of the cytoplasmic leaflet (P face) of the split axolemma reveal circular depressions that represent the secretory granule membranes fused with the plasma membrane during exocytosis. These depressions often contain granule core material in the process of extrusion into the extracellular space. The membrane surrounding some of the exocytotic openings shows a decreased number of intramembrane particles (mean diameter, 8 nm) which are elsewhere more numerous and evenly distributed on the fracture face. Endocytotic sites appear as smaller plasma membrane invaginations, with associated intramembrane particles. Moreover, such invaginations often contain large particles (mean diameter, 12 nm) that appear as clusters on *en face* views of the membrane leaflet. Quantitative analysis indicates that the number of exocytotic images increases significantly in glands from water-deprived rats. Concomitantly, the number of endocytotic figures per unit area of membrane is raised as is the number of clusters of large particles.

The observations demonstrate that, in the neurohypophysis, it is possible to distinguish exocytosis morphologically from endocytosis and that the two events can be assessed quantitatively.

KEY WORDS freeze-fracture · neurohypophysis · secretion · exocytosis · endocytosis · membrane

Several groups have now studied hormone release in the neurohypophysis by conventional electron microscopy and have concluded that it takes place by exocytosis of the secretory granules in which the hormones are stored. Images of invaginations in the axolemma, containing material of the same electron opacity as the secretory granule core, have been interpreted as the result of the incorporation of secretory granule membrane into the plasma membrane (2, 7, 17, 25, 28), but the rarity of such images has precluded quantitative evaluation and therefore correlation with stimulated hormone release. The technique of freeze-fracture, by exposing large areas of the interiors of membrane (3, 4), should overcome this difficulty. To do this, however, one must be able to recognize the membrane modifications accompanying exocytosis and, in particular, to distinguish them from those changes representative of the subsequent membrane event, endocytosis. Pre-

vious studies of freeze-fractured neurohypophyses have failed to do so (6, 8, 9, 20, 28).

In the present investigation, replicas of neural lobes from normal rats and rats in which neurohypophysial secretion was stimulated by water deprivation were used to study these phenomena further. We present different sets of observations of membrane modifications that we believe are related to exocytosis and endocytosis, respectively, and provide quantitative data that correlate these morphological changes with stimulated hormone release.

MATERIALS AND METHODS

Adult, male rats of the Sprague-Dawley strain (250–350 g body weight) were used in the present study. The animals were given food pellets (Nafag 850, Nafag Futter, Gossau, SG, Switzerland) *ad libitum*. Some animals were deprived of drinking water for either 2 or 6 days. As reported previously, this leads to marked dehydration and to enhanced neurohypophysial secretion (10, 15, 28).

The neural lobes of control and water-deprived rats were taken immediately after decapitation and immersed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2). For conventional electron microscopy, the tissues were postfixed in 2% OsO_4, stained en bloc with 1.5% uranyl acetate (dissolved in 50% ethanol), dehydrated in increasing concentrations of ethanol, and embedded in Epon. Thin sections were stained further with lead citrate. For freeze-fracture, after glutaraldehyde fixation, the glands were soaked in a 30% phosphate-buffered glycerol solution for at least 2 h. They were then placed on gold disks and quickly frozen in Freon 22 cooled with liquid nitrogen. Freeze-fracture was performed at −100°C in a Balzers BAF 301 unit (Balzers AG, Balzers, Liechtenstein) (23). The freeze-fractured tissue surface was replicated with platinum reinforced with carbon. The tissue replicas were then thawed, cleansed by digestion in NaOCl, washed in distilled water, and mounted on coated 150-mesh copper grids. They were examined with a Philips 300 microscope.

For quantitative evaluation of replicas, a total of 12 replicas of 9 control glands and 15 replicas of 11 stimulated glands were used in the following computations.

In order to assess the frequency of intramembrane particles (IMP) in each plasma membrane leaflet, at least eight pictures from different P- and E-fracture faces in each replica were taken at a constant magnification of 33,000. The area to be used for counting was selected for flatness but otherwise chosen at random over the whole replica. A calibrated square lattice was superimposed on the enlarged (3 ×) positive prints, and all particles within an area of 1 micrometer square were counted.

The number of IMP on the secretory granule membrane faces was obtained from micrographs taken at a constant magnification of 53,000 and further enlarged 3 × by printing. A small square of known area was applied to the center of the granule membrane leaflet and the particle density was derived per square micrometer.

Measurements of IMP diameter were performed with the aid of a 7 × magnifier containing a reticle calibrated in tenths of a millimeter. The width of the base of the shadow formed by the platinum-carbon covering was used to represent the particle diameter.

To estimate the number of larger membrane differentiations, such as pits and depressions per square micrometers of plasma membrane, they were counted and related to the area in which they were present, the latter measured in square centimeters with a calibrated planimeter. These evaluations were performed on micrographs with an original magnification of 33,000 and further enlarged 3× by printing. The area of membrane read in square centimeters by the planimeter was then converted to an area of square micrometers.

Student's *t* test as well as the Mann-Whitney U test were used to assess the statistical significance of all the quantitative data. In the Results section, values of IMP density are expressed as means ± SE of the mean while IMP diameters are expressed as means ± SD of the mean.

RESULTS

Conventional Electron Microscopy

In thin sections, neurohypophysial axons and axon terminals are identified by numerous dense-cored secretory granules, distributed among electron-lucent microvesicles and vacuoles. The vacuoles are often cup-shaped and similar or larger in size than the secretory granules. Exocytotic openings are seen as invaginations in the plasmalemma that contain material of the same electron density as the core of granules in the axon terminal (Fig. 1). Such images are rare, even in the glands of water-deprived rats.

Freeze-Fracture

Replicas of freeze-fractured neurohypophyses display two main aspects of the neurosecretory fibers: *en face* views of the split interiors of their plasma membranes, produced when the plane of the fracture follows the plane of the membrane, as well as views of their cytoplasmic interiors, produced when the fibers are cross-fractured (Figs. 2 and 3). The latter aspect is essential for identification, for it exposes intracellular organelles such as neurosecretory granules and microvesicles, and thus distinguishes these fibers from those of the pituicytes, the other major cells of the neurohypophysis.

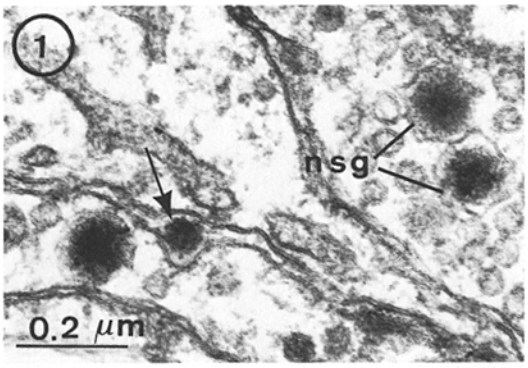


FIGURE 1 Thin section of neurohypophysial axons from a water-deprived rat. A round mass (arrow) of electron opacity similar to the core of neurosecretory granules (*nsg*) in the axon terminal fills an invagination in the cell membrane; the presence of core material identifies the invagination as resulting from the fusion of the granule membrane with the plasma membrane during exocytosis. × 70,000.

Fracture Faces of Intracellular Organelles

After fracturing, the secretory granules are split to expose either a convex or a concave face (Figs. 2 and 3). The convex fracture face represents the inner leaflet of the granule membrane (E face), adjacent to the granule core, whilst the concave face represents the outher leaflet (P face), associated with the cytoplasmic matrix. Occasionally, granules are cross-fractured, making visible a finely granular core interior (Fig. 2); the granule core surface is smooth (Fig. 3).

Intramembrane particles are found on both granule membrane leaflets but are more numerous on the P face (560 ± 39/μm^2) than on the E face (133 ± 23/μm^2). Moreover, the P-face particles are significantly larger than those on the E face (12.1 ± 3 nm and 8.0 ± 3 nm, respectively, Fig. 4).

Microvesicles are seen scattered throughout the cytoplasm (Figs. 2 and 3) or in clusters (32). They are fractured in a fashion similar to that of the neurosecretory granules, but because of their small size, we did not attempt to evaluate the number of intramembrane particles on their respective membrane leaflets. Large vacuoles, usually tubular or cup-shaped (see Fig. 14), are also evident and their P face contains large intramembrane particles (mean diameter, 11.2 ± 3 nm, n = 505).

Fracture Faces of Neurohypophysial Axon Plasmalemma

Fractures along the interior of the axon's plasmalemma expose *en face* views of either the inner P face, adjacent to the cytoplasm, or the outer E face, adjacent to the extracellular space (Figs. 2 and 3). Both membrane leaflets contain a background population of randomly distributed particles. The density of the particles on the P leaflet, (919 ± 57/μm^2), is higher than that of the E leaflet (264 ± 36/μm^2), but the mean particle diameter is similar (8.7 ± 3 nm and 8.4 ± 3 nm, respectively, Fig. 5 A and C).

An additional population of particles is present on the P leaflet (see Figs. 13 and 14). These particles, found as clusters of 6–12 particles are significantly larger than those more evenly distributed on the membrane leaflet, and show little variation in size (Fig. 5 B). They are similar in size to the intramembrane particles found on the secretory granule P face (cf. Figs. 4 A and 5 B).

Membrane Changes Related to Secretion

Exocytosis: In stimulated neurohypophyses, a recurrent image on *en face* views of the plasmalemma P face is that of depressions filled with a protruding spheroid (Figs. 6–8). The surface of the spheroid is smooth (Figs. 6–8), but, when it is partly cross-fractured, its interior appears finely granular (Fig. 7). These morphological properties are similar to those shown by granule cores still enveloped by a membrane in the axon cytoplasm (cf. Figs. 2 and 3). It is therefore likely that the protruding spheroids represent core material in the process of being released into the extracellular space (see Fig. 1). Occasional fractures that expose both the membrane and the adjacent cytoplasm reveal that the depressions represent the granule membrane continuous with the plasma membrane (Figs. 9 and 10). As in thin sections, core substance already released into the extracellular space can be seen close to the invaginations (Fig. 10). A distinctive feature of the fracture face delimiting some of the exocytotic stomata is that it contains very few or no intramembrane particles (Fig. 8).

Partial or total clearing of intramembrane particles on the fracture P face is also evident in areas of membrane forming circular elevations or "bulges" in the axon surface (Fig. 11). In the cytoplasm underlying such bulges, one may find neurosecretory granules in close proximity to the cell membrane. Images of the fracture E face (Fig. 12) also reveal granules closely applied to the plasma membrane, but viewed from the inside of the cell. As will be discussed later, the relationship

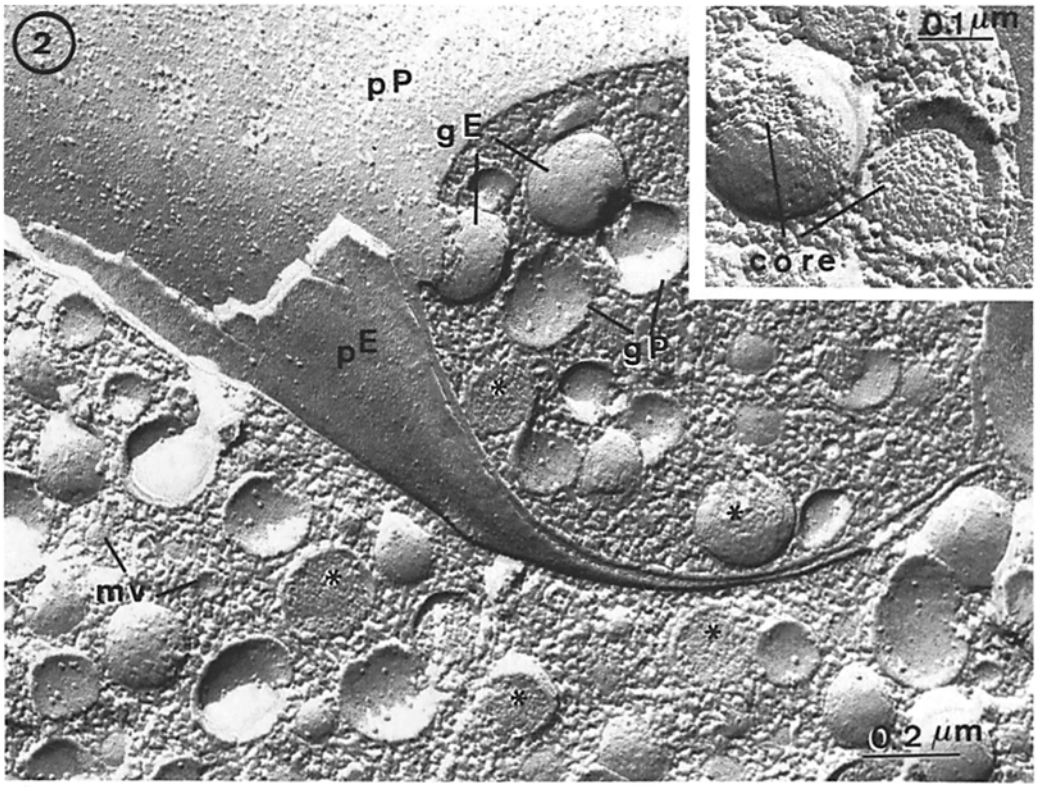


FIGURE 2 Overview of two freeze-fractured neurohypophysial axons. The fracture has exposed the interior of their plasma membranes, namely, the inner leaflet or P face (*pP*) of one axon and the outer leaflet or E face (*pE*) of the other, as well as part of their cytoplasm. Numerous neurosecretory granules and microvesicles (*mv*) serve to identify the neurosecretory fibers. The fractured secretory granule membrane shows either a concave (*gP*) or convex (*gE*) face. Both granule membrane leaflets contain intramembrane particles. When the neurosecretory granule is cross-fractured, the core substance appears finely granular (*). Note that the P leaflet of the axolemma contains more intramembrane particles than the E leaflet. × 65,000. *Inset*: Cross-fractured neurosecretory granules showing the finely granular core interior. × 100,000.

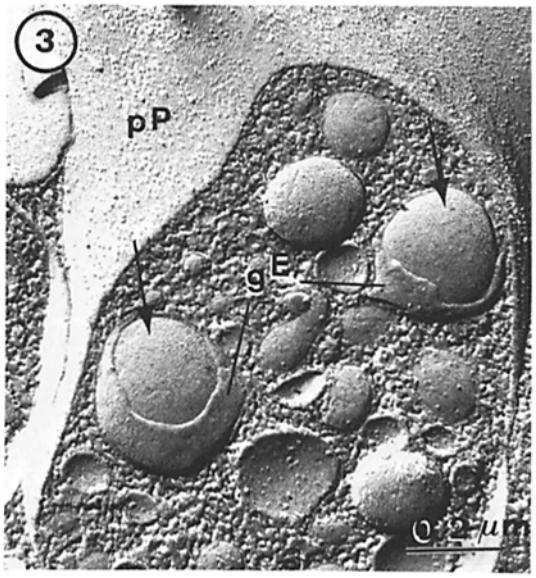


FIGURE 3 Replica of a neurosecretory axon showing part of the axolemma P face (*pP*) and part of the cross-fractured cytoplasm. The core substance of two secretory granules is shown (arrows). Unlike the granule core interior, the granule core surface is smooth. *gE*, inner granule membrane leaflet. × 61,000.

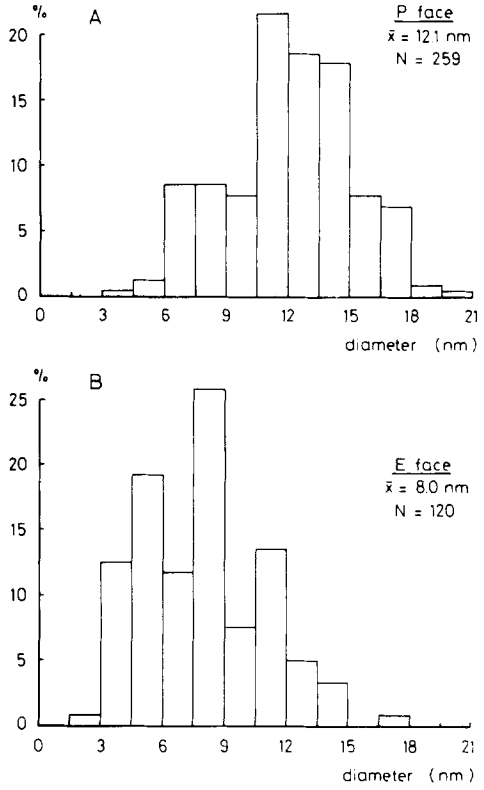


FIGURE 4 Size distribution of intramembrane particles on P and E faces of the neurosecretory granule membrane. Particles on the P face are significantly larger than those on the E face ($P < 0.001$, Student's t test).

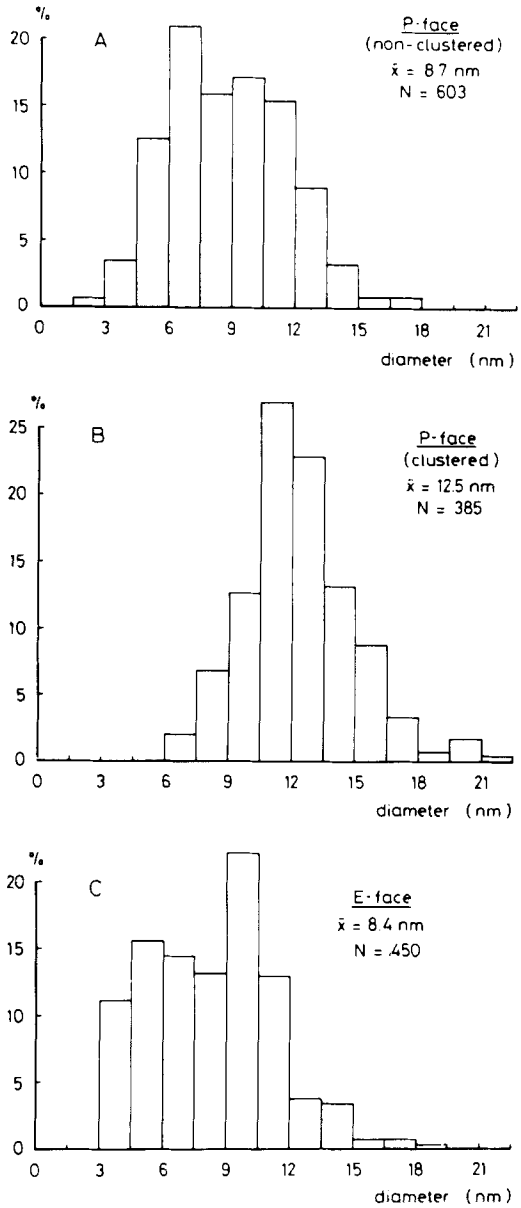


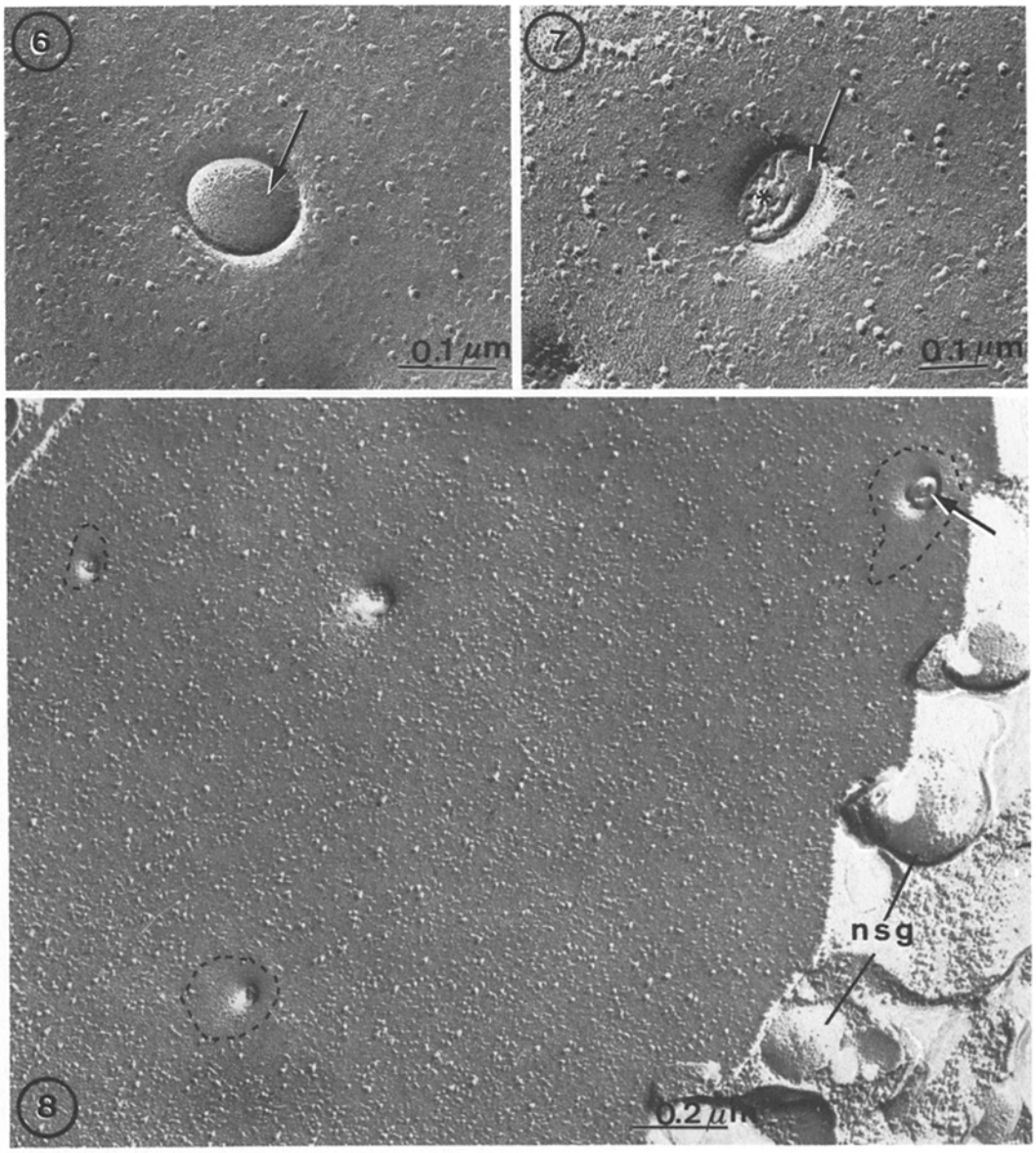
FIGURE 5 Size distribution of intramembrane particles on the P and E faces of the neurosecretory axon plasma membrane. Note that, on the P face, particles that occur in clusters have a larger mean diameter than those randomly distributed ($P < 0.001$, Student's t test).

of these particle-deficient bulges to exocytosis is not definite.

Endocytosis: Besides the membrane modifications that have been interpreted as sites of exocytosis, there are accompanying morphological changes that we believe are indicative of endocytosis. On *en face* views of the plasmalemma P face, there are many pits and depressions that appear different from those of exocytosis because they are generally smaller in diameter, contain particles and do not show core material (Figs. 13 and 14). The large clustered particles are often associated with these depressions. On the E face are numerous corresponding protuberances. Fortuitous cross-fractures exposing both the cytoplasm and the depressions reveal that the latter are invaginations of the plasma membrane which penetrate at various depths into the cell interior (Figs. 15 and 16). The P face of these invaginations is normally particulate.

Quantitative Analysis

Images representative of exocytosis, defined as invagination on *en face* views of the fracture P face showing associated granule core material, more than double in the stimulated preparations



FIGURES 6–8 Electron micrographs of membrane changes indicative of exocytosis as shown on *en face* views of the P face of neurosecretory plasmalemma.

FIGURE 6 A smooth-surfaced spheroid (arrow) is seen to fill a large circular depression in the membrane. × 130,000.

FIGURE 7 Depression in the axolemma P face containing a cross-fractured granule core; part of the smooth surface (arrow) and of the granular interior (*) of the core is visible. × 92,000.

FIGURE 8 Large area of the axolemma P-fracture face showing several exocytotic openings, one of which contains granule core material (arrow). The membrane adjacent to three openings (delineated by dotted line) is particle-poor as compared to the remainder of the fracture face. *nsg*, neurosecretory granules. × 66,000.

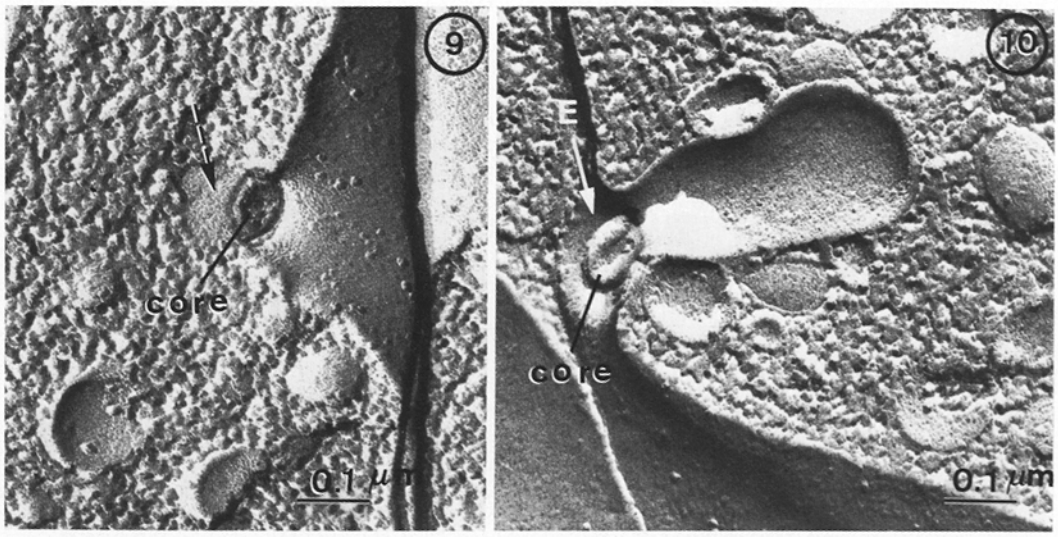


FIGURE 9 In this replica, the fracture has passed along the P face of the plasma membrane and into the cytoplasm of the same axon, thus revealing the fused granule and plasma membranes (dotted arrow). A cross-fractured granule core fills the exocytotic opening. × 92,000.

FIGURE 10 Replica in which the fracture has exposed a part of the particle-deficient granule membrane, continuous with the plasma membrane of the axon terminal. The exocytotic nature of this invagination is suggested by the presence of a granule core in the adjacent extracellular space (*E*). The surface of the granule core is smooth. × 92,000.

(Table I). A significant increase is also noted in the number of clusters of large intramembrane particles per square micrometers of membrane and in the number of particle-filled depressions (Table I).

DISCUSSION

This study has shown that it is possible to characterize membrane events taking place during and after neurohypophysial hormone release if the technique of freeze-fracture is used. Moreover, once the intramembranous changes characteristic of either exocytosis or endocytosis are differentiated morphologically, they can be assessed quantitatively and correlated with changing states of hormone release.

Exocytosis

After aldehyde and OsO_4 fixation at neutral pH (7.0–7.2), the substance contained in most neurohypophysial secretory granules appears as an electron-opaque core, detectable in thin sections (24). This property of the core substance has served to identify exocytotic profiles by conventional electron microscopy in this and previous investigations (2, 7, 17, 25, 29). The core retains its compact, electron-opaque appearance shortly after it is liberated into the extracellular space, and, since the core usually sits in a plasma membrane invagination, the latter is interpreted as the granule membrane incorporated into the cell membrane.

As in thin-sectioned material, neurosecretory granule cores can be identified in replicas of freeze-fractured neurohypophysial axons where they also serve as the main morphological markers for exocytotic figures. Such figures consist of circular depressions in the P face of the axolemma that contain material with the same freeze-fracture appearance as the core substance enclosed in the intracellular granules. In our replicas, cross-fractured core substance appears finely granular, as do the spheroids filling the depressions when the former are partly cross-fractured. Moreover, the surface of the granule core is smooth, (as shown previously by Livingston [20]), and so is the surface of the protruding masses. Similar material fills the openings of invaginations exposed when the fracture plane continues into the cytoplasm, thus identifying the depressions as the granule membrane fused with the plasma membrane.

Quantitative analysis indicates that these mem-

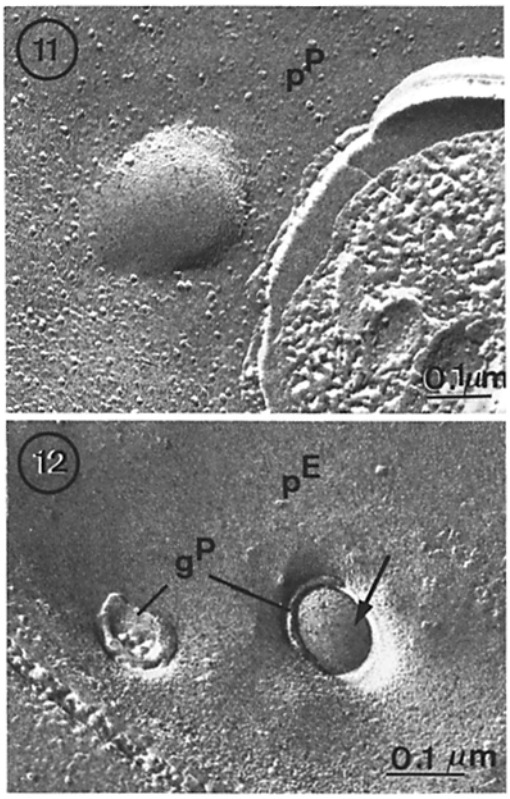


FIGURE 11 Axolemma P face (*pP*) showing a circular bulge free of intramembrane particles. The bulge suggests the presence of a neurosecretory granule closely applied to the inner leaflet of the plasma membrane. × 83,000.

FIGURE 12 Plasmalemma E face (*pE*), showing two neurosecretory granules abutting the cell surface, viewed from the inside of the cell. The membrane P face of both granules is apparent (*gP*), but in the membrane at the right it is partially covered by the smooth-surfaced granule core (arrow). × 100,000.

brane features are related to hormone release, for the number of exocytotic figures (P-face depressions with associated core material) counted on *en face* views of the membrane leaflet more than doubles in frequency in membranes from stimulated neurohypophyses. That the number of such images increases so strikingly during water deprivation is not surprising, since dehydration is a powerful stimulus for neurohypophysial secretion (28), causing a two- to threefold rise in plasma vasopressin levels as early as 12 h after the withdrawal of water, when plasma osmolarity and volume have each changed by less than 2% (10). After 2 days of water deprivation, the plasma hormone level has increased nearly 10-fold and can be sustained for several days before the neurohypophysis eventually becomes depleted (12, 15). This is permitted by a continuous synthesis of hormone in the corresponding perikarya (11, 16).

The exocytotic figures described above represent a stage in exocytosis when fusion of the secretory granule and plasma membrane has already taken place. Similar intramembrane changes associated with exocytosis were first described in freeze-fractured pancreatic islet cells (26) and in chromaffin cells of the adrenal medulla (30), although in the latter system the morphological distinction between exocytotic and endocytotic events was not clearly evident in the material presented. Earlier stages of exocytosis (i.e., apposition of granule and plasma membranes before and during fusion) have yet to be clearly characterized in the neurohypophysial axons, as they have been in other mammalian secretory cells (5, 19, 27). Data from the latter systems indicate that membrane fusion proceeds between lipid domains of the membranes, from which proteins presumably have been displaced before fusion (1, 21). These domains are visualized as areas of the fractured membrane faces devoid of intramembrane particles. In the neurohypophysial axons, such areas were detected surrounding exocytotic openings and also as bulges in the plasmalemma P face. By comparison with similar images obtained in actively secreting pancreatic islet (27) and mast cells (5, 19), the bulges could represent neurosecretory granules closely applied to the inner aspect of the axon membrane from which particles have been displaced as a prelude to fusion. Dempsey et al. (6) described similar particle-free bulges in replicas of unfixed, glycerol-treated neurohypophyses.

A definitive interpretation of such particle-poor bulges in the neurohypophysial plasmalemma is rendered difficult, however, for two reasons: (*a*) It has been shown that similar images can be produced by incomplete fixation of the tissue (22). We believe that this is not the case in the neurohypophysis, for particle-free bulges are present in perfusion-fixed material which is likely to be better preserved than tissue fixed by immersion.[1] (*b*)

[1] Such particle-free evaginations are also seen in freeze-fractured neurohypophysial axons of a hibernating rodent the garden dormouse that were fixed by perfusion. Large quantities of vasopressin are released when

Even if the particle-free evaginations are real, they need not be related to secretory granule release, for comparable images are found at neuromuscular junctions in which transmitter release has been abolished by raising the external magnesium concentration (14). For these reasons, therefore, it is not possible to definitely consider particle-deficient evaginations of the axolemma as a prefusion stage in exocytosis.

Endocytosis

In addition to randomly distributed particles (mean diameter, 8 nm), the fracture P face contains a population of large particles (mean diameter, 12 nm) that occur as clusters of 6–12 particles and often fill depressions in the membrane leaflet. Clusters of large particles in the neurohypophysial axolemma were first described in freeze-fractured glands stimulated in vitro and were believed to represent sites of exocytotic membrane fusion (8, 9). The observations presented in this study, however, suggest that they are landmarks of incipient endocytosis. Similar clusters of large particles have also been described in presynaptic membranes in the cerebellum (18) and neuromuscular junction (13, 14) where they were found associated with small dimples in the membrane P leaflet and were interpreted as sites of coated vesicle formation after synaptic vesicle discharge.

At least two possibilities exist to account for the clusters of large particles in the neurohypophysial axon membrane: (*a*) the large particles already exist in the background population of particles but they aggregate as the result of membrane changes caused by hormone release; (b) they originate in the secretory granule membrane and are added to the plasmalemma after exocytosis. We favor the latter possibility, because the large particles correspond closely in size to those found on the granule membrane P face and because their number, as clusters, doubles in the replicas of the stimulated glands. We propose, moreover, that the clusters mark areas of membrane to be reinternalized during endocytosis, since they are often associated with small-size depressions whose number also increases significantly after stimulation. In this respect, the presence of large particles in the P fracture face of the membrane limiting cup-shaped vacuoles in the axon cytoplasm (vacuoles which have been shown to be of endocytotic origin in peroxidase-tracing experiments [31, Footnote 1]) may be taken as further evidence that it is precisely those areas of the plasma membrane, containing large particles, that are due to be internalized by the axon after stimulated hormone release.

In summary, freeze-fracture of neurohypophysial axons, allows one to differentiate between the membrane changes taking place during exocytosis and those accompanying compensatory endocyto-

the rodent is awakened from hibernation, and this is reflected morphologically in a high incidence of exocytotic profiles, similar to those described in the present report. Theodosis, D. T., C. Burlet, J.-L. Boudier, and J. J. Dreifuss. 1978. Morphology of membrane changes during neurohypophysial hormone release in a hibernating rodent. *Brain Res.* In press.

FIGURES 13–16 Images of plasma membrane modifications related to endocytosis.

FIGURE 13 Among the background population of intramembrane particles on the plasmalemma P face (*pP*) are large particles, organized as clusters of 6–12 particles (circles). They are similar in size to particles found on the granule membrane P face (*gP*). Circular, particle-filled depressions are also apparent (arrows). × 60,000.

FIGURE 14 Clusters of large particles (circles) in the axolemma P face (*pP*) are also evident in this replica. At some places, they are associated with depressions in the membrane (arrow). The cross-fractured cytoplasm contains neurosecretory granules (*nsg*), large ovoid or cup-shaped vacuoles (*V*), and smaller vesicular profiles (*mv*). × 67,000.

FIGURE 15 In this replica, the fracture has exposed a large cytoplasmic vesicle opened (dotted arrow) at the cell surface (*pP*, plasmalemma P face). Since the membrane limiting the invagination contains particles, and there is no associated granule core, the vesicle is considered endocytotic. The fracture P face also contains a small circular depression (arrow) which may correspond to the opening (on *en face* view) of another endocytotic vesicle. × 65,000.

FIGURE 16 An image similar to that shown in Fig. 15, but here the invagination is tubular. Both types of invaginations could give rise to the large intracellular vacuoles shown in Fig. 14. × 82,000.

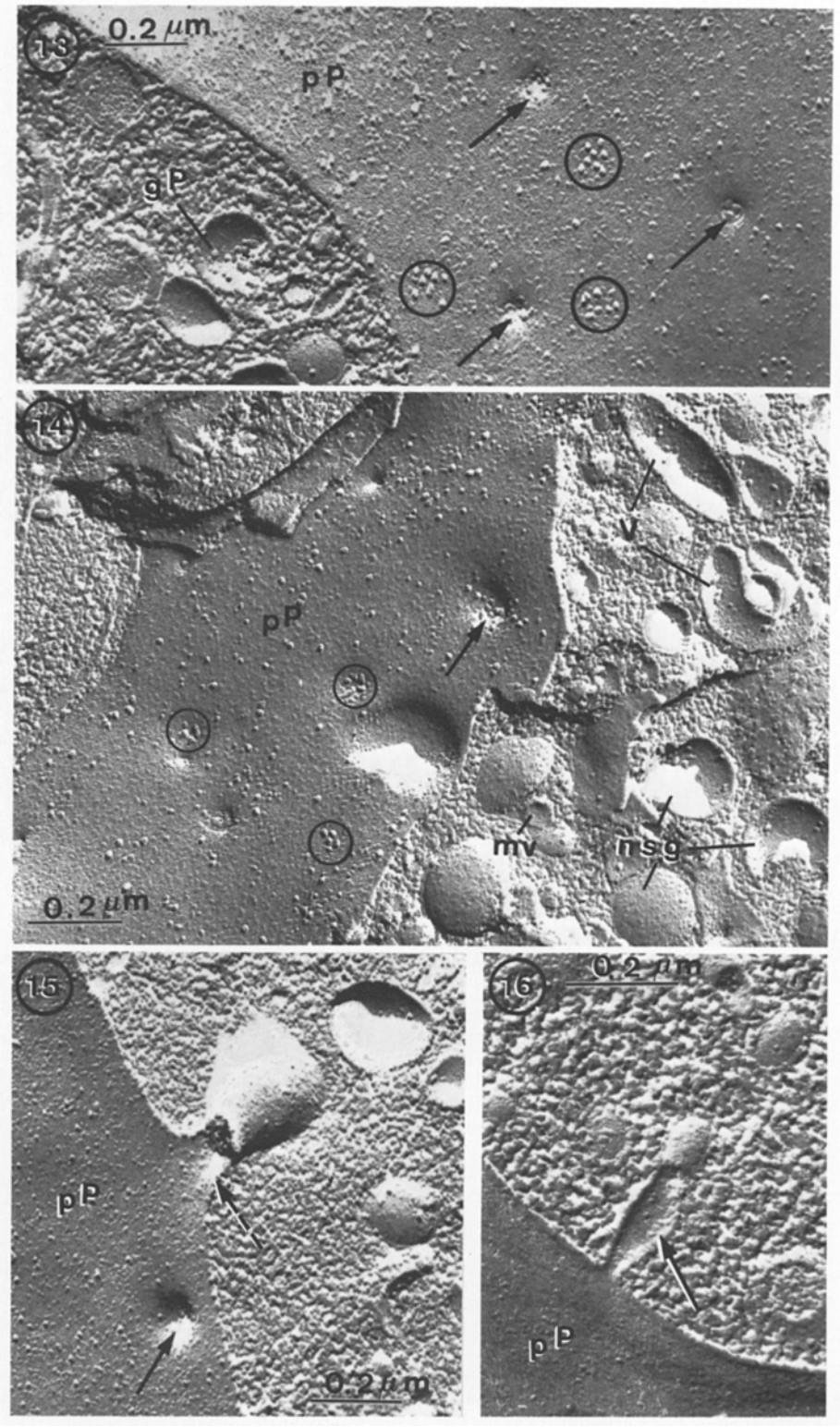

13
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TABLE I

Number of Clusters of Intramembrane Particles, Particle-Filled Depressions, and Exocytotic Images on the P Face of Plasma Membrane from Control and Stimulated Neurohypophyses

	P-face area examined	No. of clusters	No. of clusters per μm^2	No. of particle-filled depressions	No. of particle-filled depressions per μm^2	No of exocytotic stomata	No. of exocytotic stomata per μm^2
	μm^2						
Control	118.1	114	0.97*	90	0.76*	5	0.04*
Stimulated	141.1	270	1.91	159	1.13	15	0.11

Results were obtained from nine replicas of each of seven control and seven water-deprived animals.
* $P < 0.002$, Mann-Whitney U test.

sis. Exocytotic images are characterized by depressions in the axolemma that contain or are associated with secretory granule core material; smaller particle-rich depressions and clusters of large intramembrane particles identify sites of incipient endocytosis.

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