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### Research Article

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# Exocytosis in Normal Anterior Pituitary Cells

## Quantitative Correlation between Growth Hormone Release and the Morphological Features of Exocytosis

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### Abstract

We have used high-pressure freezing techniques to study exocytosis in rat anterior pituitary cells. The cells were either unstimulated or exposed to 1 nM growth hormone releasing factor (GRF) for 10 min before ultrarapid freezing. The magnitude of growth hormone (GH) release was then correlated with the number of exocytotic events observed with freeze-fracture electron microscopy.

High-pressure freezing of unfixed and uncryoprotected specimens permits cryofixation of samples up to 1 mm diam (0.5 mm thick) without ice crystal damage, and arrests exocytotic events within 10 ms. Our studies comparing conventionally fixed specimens with those prepared by high-pressure freezing confirm that areas of intramembrane particle clearing at potential exocytotic sites are an artifact of conventional fixation and/or cryoprotection techniques.

The cells exposed to 1 nM GRF released approximately fivefold more GH than did unstimulated cells. Morphologically, we have observed a 3.3-fold increase in the number of exocytotic events in GRF-stimulated cells, 33.7 events/100  $\mu\text{m}^2$  compared with 10.4 events/100  $\mu\text{m}^2$  for unstimulated cells. In additional experiments, we studied the effects of two inhibitors of GRF-induced exocytosis, somatostatin and sodium isethionate. Both compounds elicit the same response, a parallel decrease in exocytotic events and in secreted product.

We conclude that high-pressure freezing, combined with freeze-fracture and freeze-substitution processing techniques, is an excellent tool for studying the morphological aspects of exocytosis. In the present investigation, it has allowed us to quantitatively relate the biochemistry and morphology of exocytosis in anterior pituitary cells.

### Introduction

Anterior pituitary cells belong to a broad category of secretory cells that store their specific secretory products (hormones) in secretory granules and release them by exocytosis. In general terms, the exocytotic process involves apposition of the secretion vesicle against the plasma membrane, initiation of fusion of the secretion vesicle with the plasma membrane, formation of a membrane continuum, and release of the granule contents

into the extracellular space. Although both the biochemistry and morphology of exocytosis have been studied extensively in separate studies, the quantitative relationship between these processes has proven difficult to establish.

Until recently, a major problem in obtaining a quantitative correlation has been the lack of appropriate methodology for rapid fixation. Introduction of new technologies based on ultrarapid freezing of cells and tissues has resulted in a critical reevaluation of various aspects of the exocytotic process (1-5). These studies have revealed that ultrarapid freezing can reproducibly arrest transient stages of the exocytotic process without introducing many of the artifacts seen in samples exposed to chemical fixatives and/or cryoprotectants (6, 7). Thus, rapid freezing combined with freeze-fracture electron microscopy has allowed correlation of the biochemistry and morphology of exocytosis in neuromuscular junctions and chromaffin cells (3, 5). Other endocrine tissues, pituitary cells in particular, have not been examined with these new techniques. Pituitary cells with their peculiar sensitivity and responsiveness to both releasing and inhibitory hypothalamic factors (8, 9) offer a unique model for studying exocytosis.

In the present study, we have applied high-pressure freezing and freeze-fracture techniques to study exocytosis in normal anterior pituitary cells. Growth hormone releasing factor (GRF)<sup>1</sup> was used to stimulate growth hormone (GH) release, and somatostatin (SRIF) was used to specifically counteract GRF-induced exocytosis. In addition, some experiments were conducted in the presence of sodium isethionate (NaIs) which, when substituted for sodium chloride, nonspecifically inhibits exocytosis in various endocrine cells (10-12). We have observed various stages of exocytosis and have attempted to correlate the number of morphologically identifiable exocytotic events with the magnitude of GH release.

### Methods

**Materials.** HEPES, trypsin (type 1), deoxyribonuclease (type 1), BSA, and NaIs were purchased from Sigma Chemical Co. (St. Louis, MO). DME, MEM, nonessential amino acids, FCS, penicillin, streptomycin, amphotericin B, and pancreatin were obtained from Gibco (Grand Island, NY). Polyester monofilament screen, 240 mesh, was purchased from Tetko, Inc. (Elmsford, NY). Cyclic SRIF was purchased from Bachem, Inc. (Torrance, CA) and rat GRF was from Peninsula Laboratories, Inc. (Belmont, CA).

**Pituitary cell isolation and culture.** The primary buffer used to prepare and manipulate the cultured pituitary cells was  $\text{Ca}^{2+}/\text{Mg}^{2+}$  and  $\text{NaHCO}_3$ -free HBSS, containing 137 mM NaCl, 5.36 mM KCl, 0.33

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1. **Abbreviations used in this paper:** DNase, deoxyribonuclease; EF, extracellular face; GH, growth hormone; GRF, growth hormone releasing factor; IMP, intramembrane particles; NaIs, sodium isethionate; PF, protoplasmic face; SRIF; somatostatin.

mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 5.55 mM dextrose, 5 mM Hepes, 100 U penicillin/ml, and 100 µg streptomycin/ml (pH 7.4). The culture medium was DME supplemented with 10% fetal bovine serum, 0.1 mM MEM nonessential amino acids, and antibiotics (100 U penicillin/ml, 100 µg streptomycin/ml, and 2.5 µg amphotericin B/ml).

Pituitary glands were obtained from Sprague-Dawley, male rats (225–275 g) and placed in HBSS (13). The anterior lobes were cut into small fragments that were washed twice with 5 ml of HBSS. Digestion was performed with enzymes dissolved in HBSS containing 15 mM Hepes and 1.0% BSA. After initial digestion for 25 min at 37°C with trypsin (10,000 BAEE U/ml) and deoxyribonuclease (DNase, 50 Kunitz U/ml), 0.5 ml of enzyme solution per pituitary gland. The tissue-enzyme mixture was diluted with 3 vol HBSS and centrifuged at 600 g for 5 min at room temperature. The supernatant was discarded and the tissue was digested for 15 min at 37°C with 0.15% pancreatin and DNase (50 U/ml). The tissue-enzyme mixture was diluted and centrifuged as above and the supernatant discarded. The tissue fragments were broken up by trituration through Pasteur pipettes in DNase (150 U/ml). After diluting and centrifuging the cell-DNase suspension, the cell pellet was suspended in culture medium and filtered through three layers of polyester screen, 240 mesh. The resulting suspension consisted of single cells with a viability of 95% (trypan blue exclusion) and the yield was 2.5–3 × 10<sup>6</sup> cells per pituitary.

The cells (1 × 10<sup>6</sup> cells/ml) were cultured at 37°C in 12 × 75 mm sterile polypropylene tubes to which they did not attach. After 24–48 h in culture the cells from 4–8 pituitaries were centrifuged; resuspended in HBSS, pH 7.4; supplemented with 1.26 mM CaCl<sub>2</sub> · 2H<sub>2</sub>O, 0.49 mM MgCl<sub>2</sub> · 6H<sub>2</sub>O, 0.41 mM MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.5% BSA, and 5.0 mM glucose; and placed on ice until used.

In preparation for high-pressure cryofixation, 100 µl (1 × 10<sup>6</sup> cells) of the cell suspension was removed and warmed to 37°C for 30 min. The cells were centrifuged at 1,470 g for 5 min, resuspended in the same buffer containing 17% dextran (10,000 mol wt), and incubated for 5 min at 37°C. When the cells were stimulated with GRF (10<sup>-9</sup> M), it was included with the dextran. The cells were then recentrifuged (5 min at 1,470 g), the supernatant was removed and frozen (–70°C) for the assay of hormone content, and the cell pellet was resuspended in the residual buffer (5–10 µl). The cells were maintained at 37°C and loaded to the specimen holders for high-pressure freezing, as described below.

In studies with the inhibitors of exocytosis, SRIF and Nals, the cells were exposed to the inhibitor for 10 min at 37°C before removing the initial 100 µl sample.

**Ultra-rapid, high-pressure freezing.** The design of the high-pressure freezing apparatus and its operation have been recently reviewed by Gilkey and Staehelin (6). The cells were loaded by micropipette into specially designed, interlocking gold specimen cups (14) and frozen at high pressure using an HPM010 (Balzers, Hudson, NH). The time between removal of the cells from the incubation medium and freezing in the apparatus varied between 30 and 60 s. Samples that took more than 1 min to mount were discarded. Once loaded in the freezing chamber, freezing to below –50°C was accomplished in < 10 ms at 2,100 bars.

After freezing to ~ –150°C, the specimens were always kept below –100°C during the separation of the specimen holders, storage, and subsequent handling for freeze-fracture replication or freeze-substitution.

**Conventional fixation, cryoprotection, and freezing.** The freezing of glutaraldehyde-fixed cells cryoprotected with glycerol was accomplished by conventional methods (15). The cells were fixed with 2% glutaraldehyde in the supplemented HBSS buffer for 3 min at room temperature and then at 4°C for 60 min. The fixed cells were infiltrated with 30% glycerol at 4°C, concentrated by centrifugation, loaded to copper specimen supports, and frozen by plunging the loaded support into a vial of freezing dichlorodifluoromethane.

**Freeze-fracture replication.** Standard freeze-fracture procedures were employed for the preparation of freeze-fracture replicas (15–17). The fracturing and replication were performed at a temperature of

–106°C and a vacuum of < 2 × 10<sup>-6</sup> Torr in a 360-M freeze etch device (Balzers).

The replica was recovered by immersing the replicated frozen specimen in 5 ml of a viscous solution of 16% KOH in methanol at –80°C and allowing the specimen to slowly thaw as the KOH-methanol solution warmed to room temperature. This provided for the gradual evolution of gas from the specimen which, with rapid thawing, usually led to fragmentation of the replica.

The methanolic KOH was gradually replaced with distilled water, the tissue was digested with 70% H<sub>2</sub>SO<sub>4</sub> at 60°C for 90 min, and the replica was washed with water and mounted on a 400 mesh hexagonal grid without a supporting film. The replicas were observed at 80 kV in a Philips EM 300 electron microscope.

**Quantitative analysis of freeze fracture replicas.** Flat, horizontal expanses of plasma membrane replica (both extracellular face [EF] and protoplasmic face [PF]) were selected randomly and photographed at a constant magnification. Approximately 40 cells were photographed in each case such that the total amount of membrane observed was approximately equivalent for each experimental condition.

The membrane surface area evaluated in each case was measured with a computer (9835A; Hewlett-Packard Co., Palo Alto, CA) equipped with a digitizer (9874A; Hewlett Packard). The results were expressed as membrane events per 100 µm<sup>2</sup> and totaled to compare with the amount of hormone in the cell supernatant measured by radioimmunoassay.

**Freeze substitution.** The water of the frozen specimen was substituted with methanol containing 1% OsO<sub>4</sub>, 3% glutaraldehyde, 0.5% uranylacetate, and 3% water (18). 5 ml of the above substitution medium in a plastic scintillation vial was frozen with liquid N<sub>2</sub>. The frozen specimen, still in the bottom half of its freezing container, was placed specimen side up on the surface of the frozen substitution medium, and the vial was immersed in a beaker containing 200 ml of liquid N<sub>2</sub>. The beaker was placed in a styrofoam box containing dry ice, and the specimen was allowed to come to room temperature in 3 d. After an additional hour at room temperature, the specimen was removed from the gold holder, the methanol was replaced with anhydrous acetone, and the specimen was embedded in Spurr's plastic.

## Results

Our initial experiments were designed to assess the magnitude of GH release under basal and stimulatory conditions in the absence and in the presence of two inhibitors of exocytosis, specifically, SRIF and Nals. The exposure of anterior pituitary cells to 1 nM GRF resulted in a rapid increase in GH release (Table I). It went from 236 ± 12 ng/10 min to 1,458 ± 124 ng/10

Table I. GH Release from Anterior Pituitary Cells

Agent used	GH release ng/10 min
1 Control	236 ± 12
2 SRIF 10 <sup>-6</sup> M	150 ± 8*
3 Nals, 120 mM	185 ± 15
4 GRF, 1 nM	1,458 ± 124*
5 GRF with SRIF	130 ± 10‡
6 GRF with Nals	356 ± 31‡

Results are presented as mean ± SEM of five experiments.

\* P vs. control < 0.01.

‡ P vs. GRF alone < 0.001.

GRF 1 nM, SRIF 0.1 µM, Nals 120 mM.

min,  $P < 0.001$ . SRIF, a specific inhibitor of both basal and GRF-induced GH release (9), reduced basal GH output to  $150 \pm 8$  ng/10 min and completely eliminated GRF-induced exocytotic GH release ( $130 \pm 10$  ng/10 min). The action of SRIF was dose-dependent, with  $IC_{50}$  observed at a concentration of  $5 \times 10^{-11}$  M for both basal and GRF-induced GH release. The maximal effect of SRIF was seen at a concentration of  $10^{-7}$  M.

In parallel experiments, anterior pituitary cells were incubated with and without GRF in the presence of NaIs. Isethionate is an impermeant anion that, when substituted for chloride, inhibits exocytotic hormone release. Its action is nonspecific and has been demonstrated in numerous endocrine tissues (10–12). NaIs exerted only a minimal effect on basal GH release (a decrease from  $236 \pm 12$  ng/10 min to  $185 \pm 15$  ng/10 min,  $P < 0.05$ ), but significantly reduced GRF-induced

exocytotic release of GH from  $1,458 \pm 124$  ng/10 min to  $356 \pm 31$  ng/10 min ( $P < 0.001$ ).

The cells from these experiments (control, SRIF-treated, and NaIs-treated) were rapidly frozen under high pressure for the morphological examination and quantitation of exocytosis.

Figs. 1–4 illustrate the morphology of anterior pituitary cells ultrarapidly frozen under high pressure. Fig. 1 is a freeze-fracture replica of a cross-fractured pituitary cell. The smooth, turgid appearance of the plasma, nuclear, and vesicle membranes on this and subsequent micrographs of high-pressure frozen cells is indicative of excellent freezing with no ice crystal damage.

The intricate configuration of the Golgi apparatus as preserved by high pressure-freezing is revealed in Fig. 2 A, which is a thin section image of a freeze-substituted cell. When com-

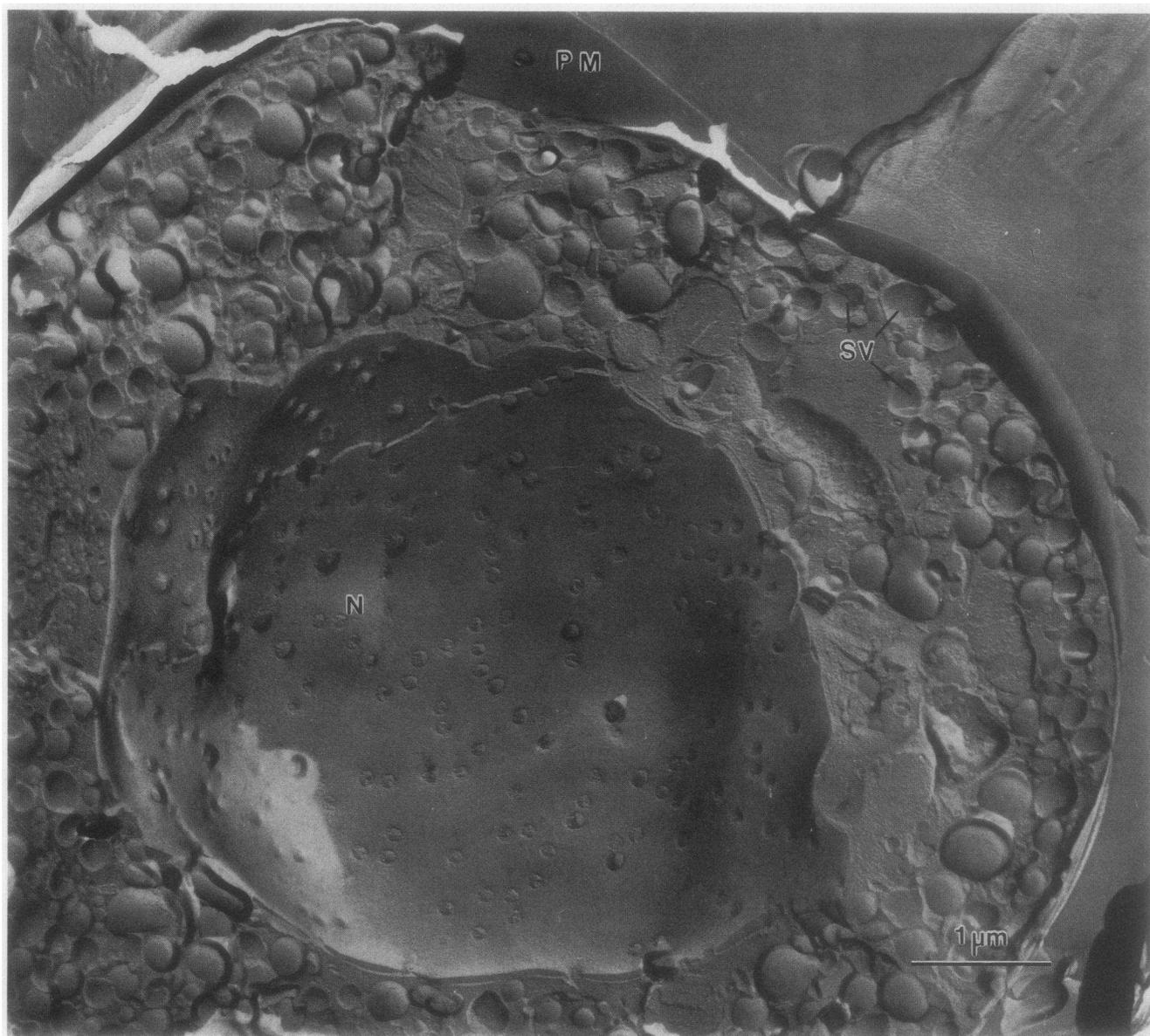
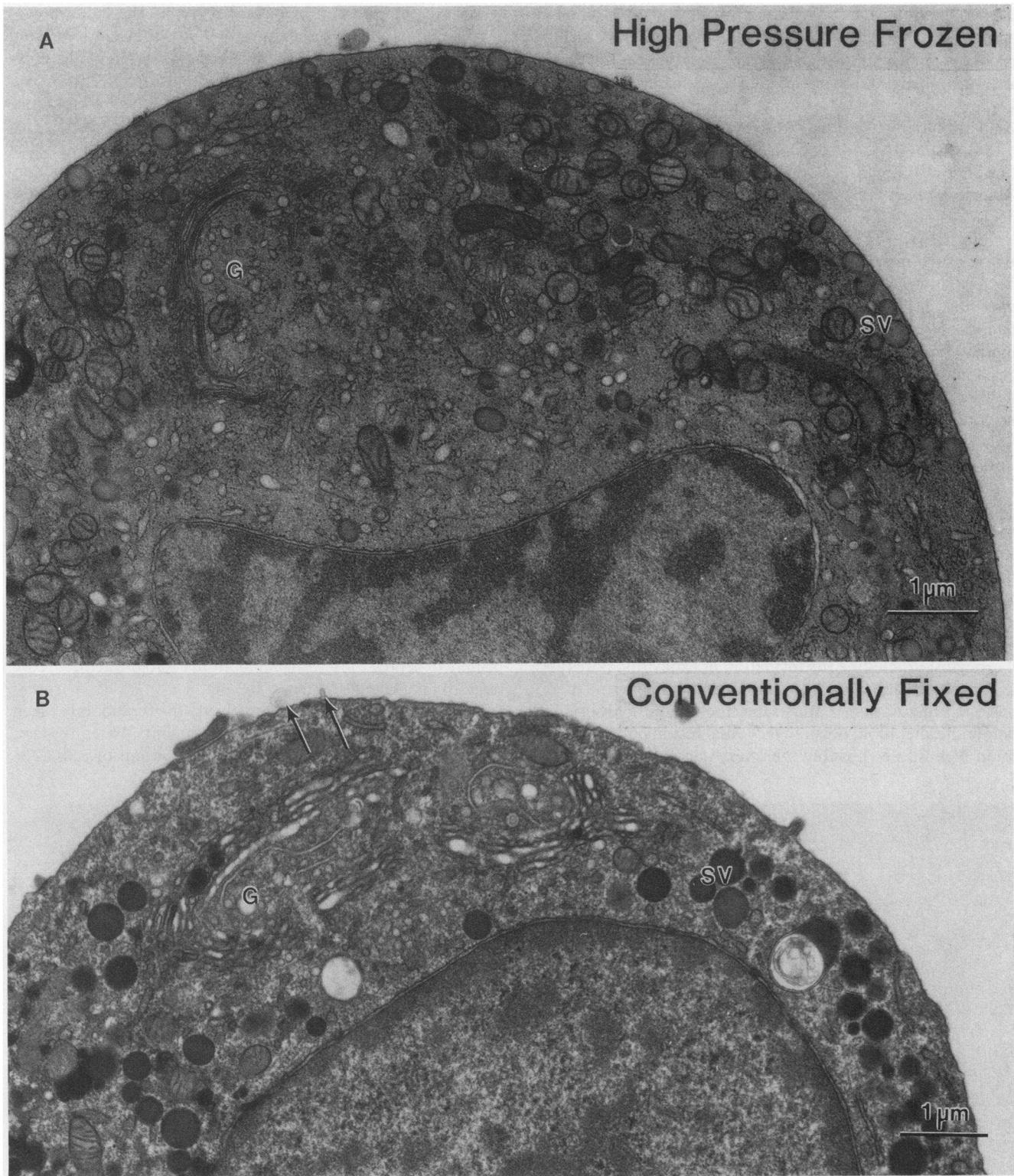


Figure 1. Freeze-fracture replica of a pituitary cell ultrarapidly frozen at high pressure. Labeled are the nuclear membrane (N), plasma membrane (PM), and secretory vesicles (SV). The excellent freezing is indicated by the smoothness of all types of membranes. Bar, 1  $\mu$ m.



**Figure 2.** Comparison of the morphology of a pituitary cell ultrarapidly frozen at high pressure and a cell chemically fixed by conventional means. (A) Cell frozen at high pressure and freeze-substituted (see Methods). The excellent quality of the freezing and structural preservation is evidenced by the smoothness of the membranes, particularly the plasma membrane. The nucleoplasm, which is particularly prone to the formation of ice crystals, also exhibits a very fine texture indicating a lack of ice crystal formation. The tight packing of the Golgi cisternae and the large number of small vesicles in the vicinity of the Golgi apparatus seen in the high-pressure frozen cell

highlight some of the more subtle shortcomings of chemical fixatives such as swelling of Golgi cisternae, etc. (B) Cell fixed with glutaraldehyde and dehydrated conventionally. Note the relatively swollen cisternae of the Golgi. Also note that there are fewer small vesicles in the vicinity of the Golgi apparatus and that there is less retention of cytoplasmic ground substance. The plasma membrane is more irregular than that of the cryofixed specimen. Also evident are small blisters on the plasma membrane (arrows). Labeled are the Golgi apparatus (G) and secretory vesicles (SV). Bars, 1  $\mu$ m.

pared to the Golgi of a conventionally fixed and thin-sectioned sample (Fig. 2 *B*), it is apparent that the Golgi cisternae of specimens prepared by high-pressure freezing are not as dilated or swollen as those of glutaraldehyde-fixed cells, and that they are also surrounded by many more very small vesicles. A comparison of Figs. 2 *A* and *B* also indicates a greater retention of cytoplasmic ground substance and a much more defined distribution of organelles with specific dimensions and morphologies in high-pressure frozen and freeze-substituted specimens.

Rare views of cross-fractured vesicles captured in the process of exocytosis by high-pressure freezing are illustrated in Figs. 3 *A* and *B*, with the earliest stage shown in Fig. 3 *A* and a latter stage at Fig. 3 *B*. Fig. 4 *A* shows a PF view of the plasma membrane of a pituitary cell treated with 1 nM GRF for 10 min and rapidly frozen under high pressure. Note the smooth appearance of the plasma membrane, the random distribution of IMPs, and the distinct configurations of the secretory vesicle fusion/discharge sites. These rapidly frozen cells also lack any evidence of clearing of IMPs in regions of possible sites of exocytosis as is typically observed in glutaraldehyde-fixed and glycerinated samples (Fig. 4 *B*). Thus, our micrographs of high-pressure frozen cells support previous observations made from other types of ultrarapidly frozen secretory cells that IMP clearing at the site of exocytosis (19) is not an obligatory early stage of exocytosis, and that particle clearing (Fig. 4 *B*) is an artifact of conventional fixation/glycerination specimen preparation methods (1–5, 20).

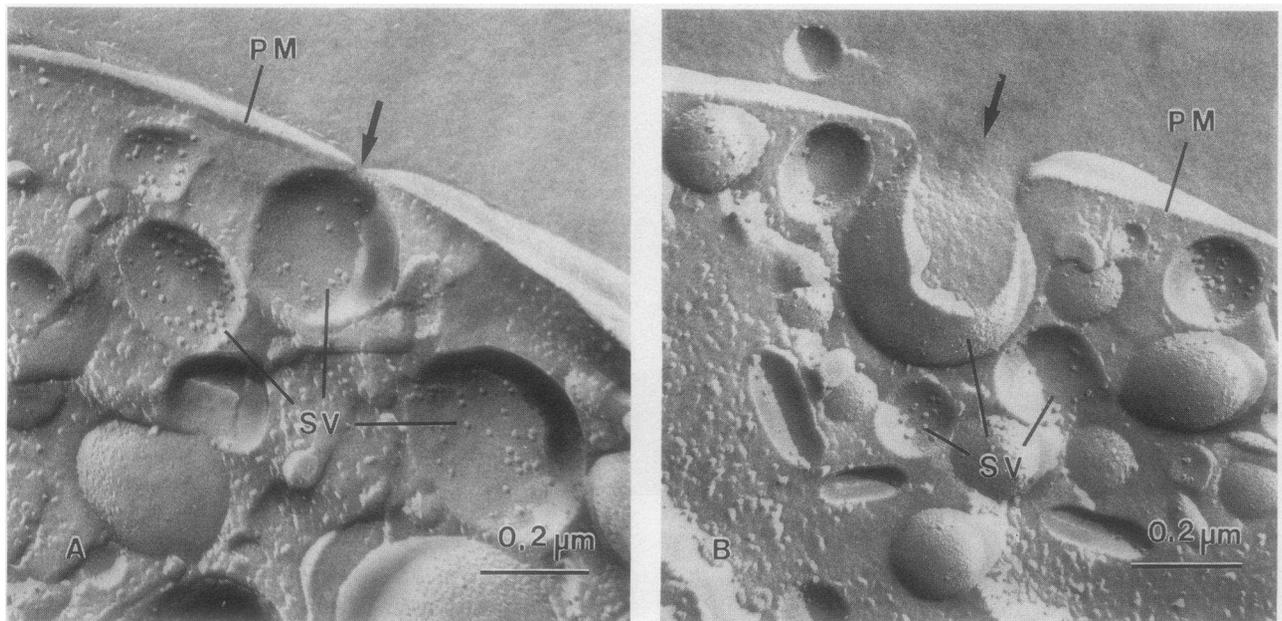
The numbering shown in Fig. 4 *A* points out various stages of exocytosis, including the formation of exocytotic pores and the integration of the vesicle membrane into the plasma membrane. Configuration 1 illustrates the smallest observable membrane alteration, possibly the initial fusion pore. Configuration 6 illustrates possibly the latest stage of integration of

vesicle membrane seen in this micrograph. The flatter membrane depressions (configuration 6), postulated to be later stages of membrane integration, are characterized by the presence of larger than average and uniformly sized PF IMPs that are typical of secretion vesicles (compare Figs. 3 *A* and 4 *A*). Similar membrane configurations can also be discerned in Fig. 5, which depicts an EF view of the plasma membrane of a pituitary cell. Also seen in Fig. 5 is a typical tight junction network that is retained by these cells during their isolation and culture.

To quantitate the morphological events associated with exocytosis, we have compared en face views of freeze-fractured plasma membranes from stimulated and nonstimulated cells. Both PF and EF plasma membrane faces were included in the quantitation and were exploited for the interpretation of exocytotic events. In PF views, the membrane features counted as presumptive exocytotic or endocytotic events in each photograph were identified as small dimples (10–200 nm diam). One characteristic class of membrane configurations included depressions with a central region free of IMPs, representing the contents of the secretory granule. A second class consisted of shallow pits containing only large PF IMPs similar to those of secretory granules (see Fig. 4 *A* for example). The second class may represent the terminal stages of exocytosis or sites of incipient endocytosis (21, 22).

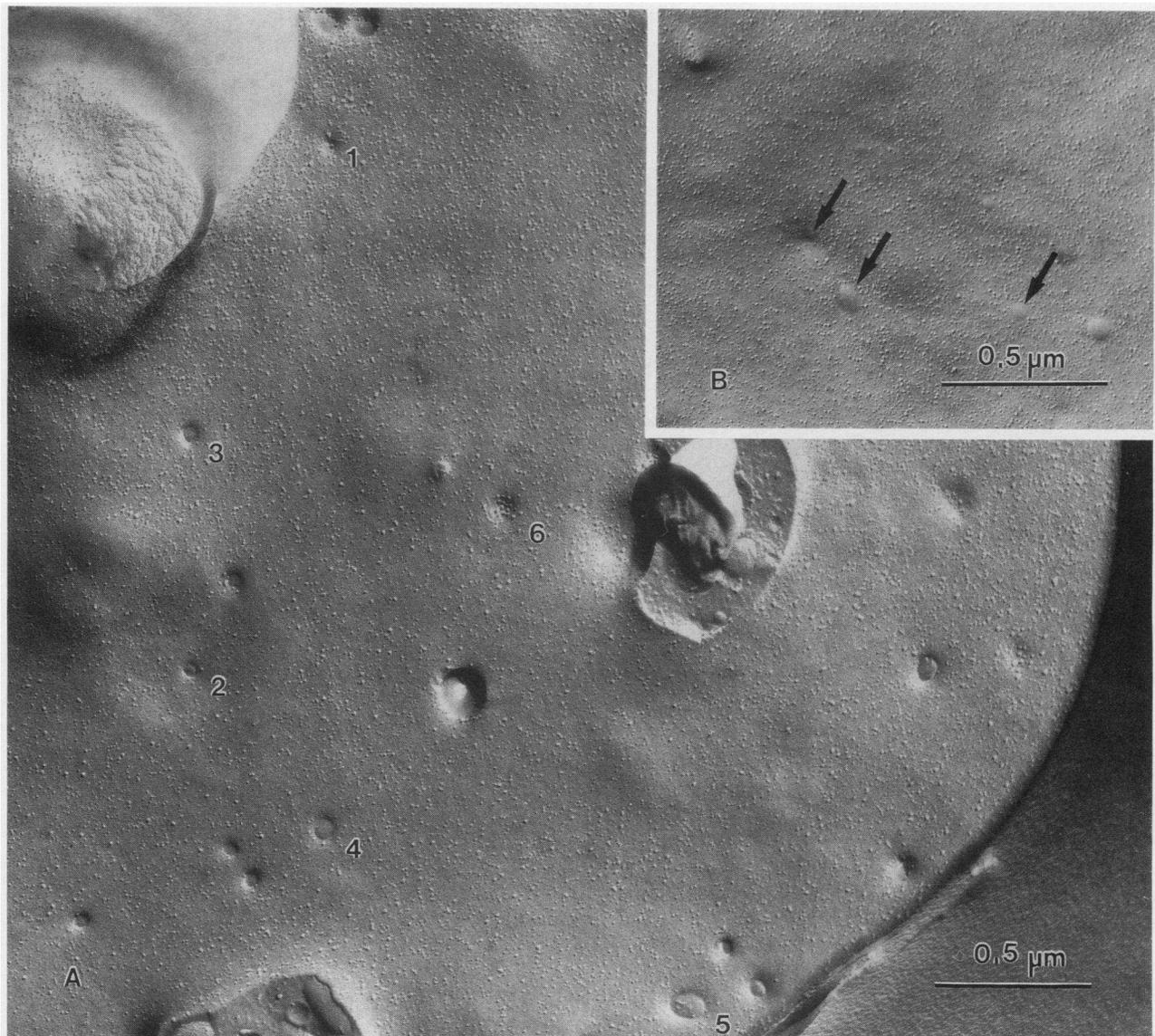
In EF views of the plasma membrane, exocytotic pores usually appear as round stalks or small volcano-like configurations (Fig. 5), which may or may not be transversely fractured. Projections from the cell surface, such as microvilli, appear as rounded depressions with flat bottoms (cross-fractured core region) and have diameters > 0.2  $\mu\text{m}$ .

The quantitative analysis involved 40–50 randomly taken photographs of cells from each experiment. An equivalent, total surface area was analyzed for each group of cells. The



**Figure 3.** Pituitary cells treated with 1 nM GRF for 10 min and frozen at high pressure. (A) Secretory vesicle captured at an early stage of fusion with the plasma membrane (PM) and displaying a single narrow exocytotic pore (arrow). The secretory vesicles (SV) are identified by the relatively few but large IMPs on their membrane PF.

There are no distortions of the plasma membrane other than the pore at the point of pore formation. (B) Secretory vesicle at an advanced stage of exocytosis (arrow). Again, there is no further distortion of the plasma membrane other than its inward turning at the site of pore formation. Bars, 0.2  $\mu\text{m}$ .



**Figure 4.** PF of the plasma membrane of pituitary cells treated with 1 nM GRF for 10 min. (A) Cell frozen at high pressure. The membrane shows a random and homogeneous distribution of IMPs. Exocytotic pores are seen as invaginations of the membrane with a central area free of IMPs. This central area represents the contents of the secretory vesicle. The numbers point out various stages of exocytotic events associated with vesicle-mediated secretion, starting with the smallest observable pore (1) and proceeding to a membrane depression (6). (The difficulty in distinguishing between exocytotic and en-

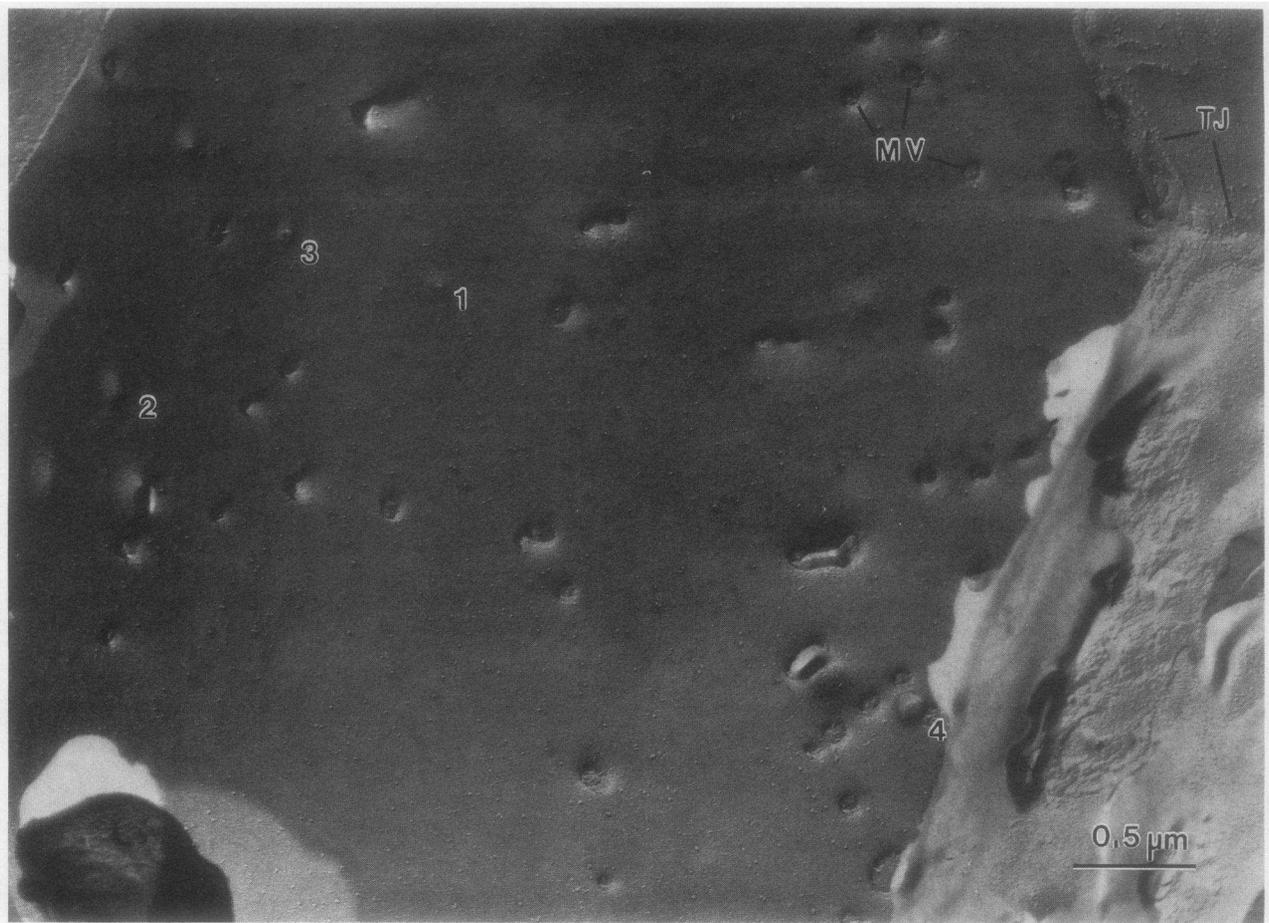
docytotic events is discussed in the text.) These putative final membrane depressions are characterized by IMPs of a size characteristic of those of secretion vesicles (Fig. 3 A). Note that the size and distribution of the IMPs are the same in the vicinity of the pore as they are throughout the rest of the membrane. (B) Cell frozen after fixation with glutaraldehyde and cryoprotected with glycerol. This replica reveals clearing of IMPs from possible sites of exocytosis (arrows), an artifact of the specimen preparation steps before freezing. Bars, 0.5  $\mu$ M.

exocytotic pores on the flat areas of the plasma membranes were counted and morphometric analysis was then performed to measure the surface area in the counted membrane region. The number of exocytotic events observed in these photographs is expressed /100  $\mu$ m<sup>2</sup> of area of plasma membrane.

The data from 89 control (unstimulated) and 84 GRF-stimulated cells (Fig. 6) revealed that the cells can be arbitrarily divided into three subgroups. In the group of unstimulated cells, the first subgroup ( $n = 48$ , 54%) exhibited no exocytotic events. The second subgroup ( $n = 27$ , 30%) showed 5–25 exocytotic events /100  $\mu$ m<sup>2</sup> cell surface (“slow-secreting” cells), whereas the third and smallest subgroup ( $n = 14$ , 16%) pos-

sessed > 25 events /100  $\mu$ m<sup>2</sup> cell surface. The majority of unstimulated cells (84%) showed either no or relatively few exocytotic events.

After the cells were challenged with 1 nM GRF, the composition of these three subgroups changed: 14% (12 cells) exhibited no obvious exocytotic events, 27% of the cells ( $n = 23$ ) revealed low numbers of exocytotic events (5–25 events /100  $\mu$ m<sup>2</sup> cell surface), and 56% of the cells ( $n = 45$ ) possessed high numbers of exocytotic events (> 25 events /100  $\mu$ m<sup>2</sup> cell surface). These morphological findings are consistent with the presence of ~ 50% of GRF responsive cells in the primary culture of anterior pituitary cells (23).



**Figure 5.** The EF of the plasma membrane of a pituitary cell frozen at high pressure. The exocytotic sites appear as rounded stalks or small, volcano-like projections. The possible sequence of events associated with the formation and enlargement of exocytotic pores is indicated by numbers 1–4. Also labeled are microvilli (*MV*) and a tight junction (*TJ*). Bar, 0.5  $\mu\text{m}$ .

The results of the parallel determinations of GH release and morphological evidence for exocytosis are summarized in Fig. 7. We have observed approximately a 3.3-fold difference in the number of exocytotic events between GRF-stimulated (33.7 events /100  $\mu\text{m}^2$ ) and unstimulated cells (10.4 events /100  $\mu\text{m}^2$ ). This correlates remarkably well with the biochemical data which demonstrated a 5–6-fold increase in GH secretion. Both inhibitors of exocytosis completely eliminated the GRF-induced increase in the number of morphologically identifiable exocytotic events (Fig. 8). In the presence of either SRIF or NaIs, we observed no difference in the number of exocytotic events between unstimulated and GRF-stimulated cells.

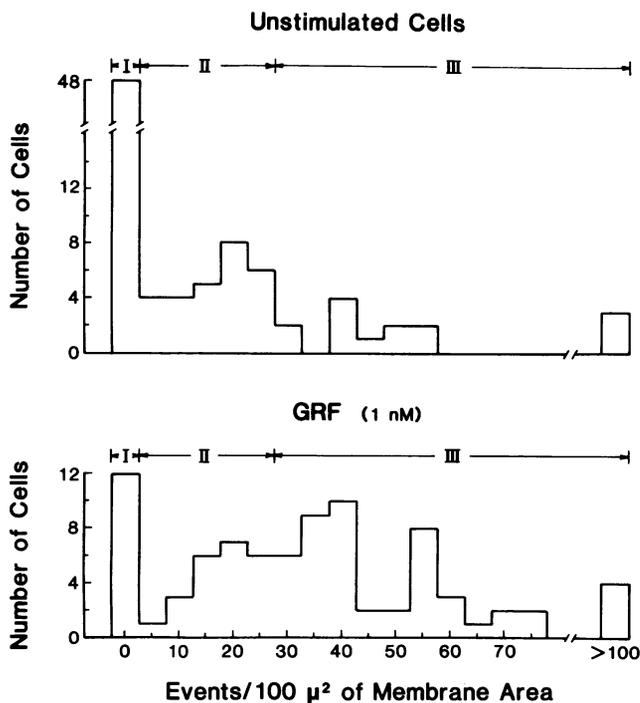
## Discussion

The two main goals of this study were: (a) to evaluate the potential of high-pressure freezing for studying transient changes in the plasma membrane of anterior pituitary cells associated with exocytosis, and (b) to obtain a quantitative correlation between the amount of secretion as measured by biochemical techniques and the number of exocytotic events associated with a given rate of secretion controlled by specific releasing and inhibitory factors.

The theory and technique of high-pressure freezing have

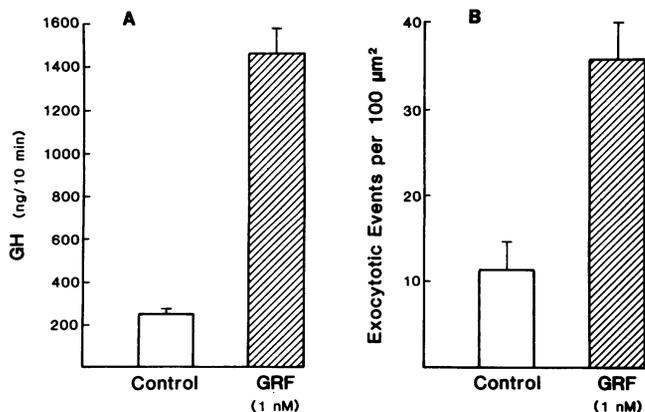
been recently reviewed by Gilkey and Staehelin (6). Like all ultrarapid freezing techniques currently employed for the preservation of cellular ultrastructure, high-pressure freezing is capable of stabilizing biological specimens two orders of magnitude faster than even the very best chemical fixatives, thereby providing researchers the opportunity for studying transient cellular phenomena more accurately. The advantage of high-pressure freezing over slam-freezing and propane-jet freezing techniques is that it permits vitrification of samples up to 0.6 mm thick, vs. a maximum depth of good freezing of 40  $\mu\text{m}$  for specimens frozen under atmospheric conditions. Previous studies of high-pressure frozen nerve (24), liver (25), cartilage (26), and root tip (27) cells have been mostly of the survey type and have not focused on a specific cellular activity such as exocytosis. They have shown, however, that the transient (20 ms) exposure to high pressure has little, if any, effect on cell membranes and microtubules. Using this technique, we routinely obtained specimens with excellently frozen cells. Without dextran, we have obtained ~5% of excellently frozen specimens containing cells with no ice crystal damage. With the dextran, we were able to increase this figure to 35–45% of specimens. Also, the presence of dextran increased the number of excellently frozen cells per specimen.

Our micrographs (Figs. 1–5) demonstrate that high-pressure freezing can be used successfully to obtain superiorly pre-

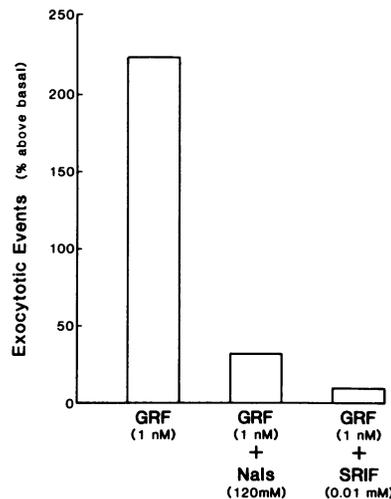


**Figure 6.** Histogram of 89 unstimulated and 84 GRF-stimulated cells. The cells were frozen under high pressure and their freeze-fracture replicas examined for the presence of morphologically identifiable exocytotic events. The results are expressed /100 μm<sup>2</sup> cell surface. Three subgroups of cells can be clearly identified: I, no exocytotic events; II, slow-secreting cells (5–25 exocytotic events /100 μm<sup>2</sup> cell surface); III, actively secreting cells (more than 25 exocytotic events /100 μm<sup>2</sup> cell surface). These categories are indicated by the arrows. A comparison of unstimulated and GRF-treated cells is provided. See Results for details.

served pituitary cells for ultrastructural analysis both by means of freeze-fracture and freeze-substitution techniques. Probably the most notable differences between high-pressure frozen, and chemically fixed cells that can be attributed to the better preservation obtainable by high-pressure freezing, are the presence of unexpectedly large numbers of very small vesicles in and around the Golgi apparatus, the lack of swelling of Golgi cisternae, and the distinct spatial organization of ER cisternae



**Figure 7.** GH release (A) and number of exocytotic events (B) in control (unstimulated) and GRF-stimulated cells. Results are expressed as mean ± SEM.



**Figure 8.** The number of exocytotic events per 100 μm<sup>2</sup> of cell surface expressed as a percentage above the number of exocytotic events in unstimulated cells. The cells were exposed to 1 nM GRF alone, and GRF in the presence of either SRIF (10<sup>-5</sup> M) or Nals (120 mM).

throughout the cytoplasm with alternating dilated and constricted regions (compare Figs. 2, A and B). In addition, the superior preservation of the plasma membrane permits a clear distinction to be made between postulated exocytotic/endocytotic events and other types of membrane configurations (Figs. 4 and 5). Our findings also lend support to the contention that areas of IMP clearing at potential exocytotic sites are artifacts of conventional fixation and processing techniques (1–5, 20), and that they are not genuine hallmarks of structural events associated with exocytosis as previously postulated by a number of research groups (19).

Figs. 4 and 5 demonstrate that high-pressure freezing can preserve the same intermediate stages of vesicle-mediated secretion as previously demonstrated in very thin samples with slam and propane-jet freezing techniques. These include establishment of the initial contact between secretion vesicle and plasma membrane, focal fusion leading to the formation of a single exocytotic pore, the enlargement of this pore into a full opening or “Omega figure”, and the eventual flattening of the secretion vesicle membrane in the plane of the plasma membrane (Figs. 3–5). It is not clear what serves as a mediator of contact between the secretory vesicle and the plasma membrane, but the inward dimpling of the plasma membrane at the site of contact and focal fusion suggests the presence of some kind of linking structure (4).

As is evident from the above considerations and from Figs. 6–8, the rapid rate of tissue stabilization that can be achieved with high-pressure freezing, and the excellent structural preservation of anterior pituitary cells, opens up the possibility of correlating structural and biochemical parameters of secretion in these cells. Comparable correlative studies of secretion have previously been reported for neuro-muscular junctions (3) and for chromaffin cells (5). However, in both of these systems only the effects of stimulatory conditions were investigated. The present study covers both stimulatory and inhibitory conditions of secretion. As seen in Fig. 7, the number of postulated exocytotic figures rises and falls in parallel with the amount of product that is secreted, close to a fivefold increase in secreted GH being matched by roughly a threefold increase in exocytotic membrane configuration. This confirms previous observations suggesting that GH secretion by anterior pituitary cells is brought about by vesicle-mediated exocytosis. The data are also consistent with the data from the neuromuscular junction

and chromaffin cell studies listed above (3, 5). These studies reported that vesicle-mediated secretion involves the quantal discharge of secretory products, thus leading to a linear relationship between number of secretory events and amount of secretion.

It should be noted that some limitations still apply to the current study. Thus, the pituitary gland is a heterogeneous tissue comprised of various types of secretory cells, some of which are clearly not responsive to GRF. The fact that we achieved a close correlation between the number of exocytotic events and the amount of GH released may have been somewhat fortuitous in that the nonresponsive cells did not greatly affect the overall results. Another potential problem related to the morphological findings is the difficulty of distinguishing between exocytotic and endocytotic events. If endocytosis-related membrane recycling occurred at a fairly constant rate compared with the rate of secretion, then the number of endocytotic events counted as exocytotic would be proportionally greater under basal or inhibitory conditions than under stimulatory conditions. Thus, our quantitative morphological data would tend to underestimate the difference in exocytotic structures between stimulated and unstimulated cells. This could explain the somewhat greater proportional increase in secreted product versus exocytotic events illustrated in Fig. 7.

Nevertheless, it is apparent that the use of the ultra-rapid freezing technique afforded a unique opportunity to observe and characterize various facets of the exocytotic hormone release in anterior pituitary cells. It was possible to define a close quantitative relationship between the number of cellular exocytotic events and changes in the magnitude of GH release from the cells subjected to diverse experimental manipulations.

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