Number of Secretory Vesicles in Growth Hormone Cells of the Pituitary Remains Unchanged After Secretion

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Immunogold-labeled transmission electron microscopy (TEM) was used to determine the total number of secretory vesicles in resting and in growth hormone (GH)–stimulated porcine pituitary cells. We identified three categories of vesicles: filled, empty, and partly empty. Resting GH cells contained more than twice as many filled vesicles than did the stimulated ones. Stimulated cells, however, contained nearly twice as many empty vesicles and 2.5 times more partly empty vesicles than did resting cells. Secretory vesicles in GH cells further revealed the localization of GH only in electron-dense vesicles in both resting and stimulated cells. The total number of secretory vesicles did not change after secretion. These results are consistent with a mechanism that, after stimulation of secretion, vesicles transiently dock and fuse at the fusion pore to release vesicular contents. Exp Biol Med 229:632–639, 2004

Key words: GH cells; secretory vesicles; exocytosis

Physiological processes such as neurotransmission and the secretion of enzymes or hormones require fusion of membrane-bounded secretory vesicles at the cell plasma membrane and the consequent release of vesicular contents. It has been commonly accepted that exocytosis requires the total incorporation of secretory vesicle membrane into the cell plasma membrane for release of vesicular contents; however, studies in the last decade (1–9) clearly demonstrate otherwise. Earlier transmission electron microscopy (TEM) studies on mast cells demonstrate that, after stimulation of secretion, several intact as well as empty and partly empty secretory vesicles are present (7). Quantitative TEM on stimulated and resting bovine chromaffin cells of the adrenal cortex showed no significant change in the number of peripheral dense-core vesicles after stimulation of secretion (2). Similarly, combined studies using atomic force microscopy and TEM clearly demonstrate no change in the total number of secretory vesicles following secretion in pancreatic acinar cells (10). Fusion pores or depressions in pancreatic acinar- or GH-secreting cells are cone-shaped structures at the plasma membrane, with a 100- to 150-nm diameter opening (3, 6). Membrane-bounded secretory vesicles ranging in diameter from 0.2 to 1.2 μm dock and fuse at depressions to release vesicular contents. After fusion of secretory vesicles at depressions, a 20%–40% increase in depression diameter has been demonstrated. It has therefore been concluded that secretory vesicles "transiently" dock and fuse at depressions (11). In contrast to accepted belief, if secretory vesicles were to completely incorporate at depressions, the fusion pore would distend much wider than observed. In addition, if secretory vesicles were to completely fuse at the plasma membrane, there would be a decrease in total number of vesicles after secretion. Examination of secretory vesicles within cells before and after secretion in pancreatic acinar cells (10) demonstrates that, although the total number of secretory vesicles remains unchanged after secretion, the number of empty and partly empty vesicles increases significantly, supporting the occurrence of transient fusion (10). Earlier studies on mast cells also demonstrated an increase in the number of spent and partly spent vesicles after stimulation of secretion without any demonstrable increase in cell size (1). Other supporting evidence favoring transient fusion is the presence of neurotransmitter transporters at the synaptic vesicle membrane. These vesicle-associated transporters would be of little use if vesicles were to fuse completely at the plasma membrane to be endocytosed at a later time. Although the fusion of secretory

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vesicles at the cell plasma membrane occurs transiently, complete incorporation of membrane at the cell plasma membrane takes place when cells need to incorporate signaling molecules like receptors, second messengers, and ion channels (11). Therefore, in GH-secreting cells, transient fusion is indicated and no change in total number of vesicles is hypothesized. This study has been undertaken to test this hypothesis.

Materials and Methods

Experimental Animals. Neonatal Yorkshire male and female pigs (n = 4), 1–3 days of age, from the Iowa State University Animal Nutrition Farm were anesthetized with injections of pentobarbital sodium (Abbott Laboratories, North Chicago, IL) and decapitated. Pituitary glands were immediately removed to dental wax and cut sagittally in half. Animal care and experimental protocols were in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.

Tissue Preparation. The neural and intermediate lobes of the pituitary were removed. The anterior lobe was cut into cubes of less than 1 mm on dental wax, and each sagittal half of the gland was exposed to equal volumes (1 ml at 25°C) of phosphate-buffered saline (PBS) or the GH secretagogue, L-692,585 (Merck Research Laboratories, Rahway, NJ), 20 mM, in PBS for 90 secs. The reaction to the secretagogue was stopped by washing the tissue three times with 1 ml PBS and was immediately followed by tissue fixation for TEM (Fig. 1).

TEM Methods to Optimize Structure. The minced pituitary gland exposed to PBS or L-692,585 was fixed at 2°C in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) and 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) 2°C for 2 hrs and then transferred to 1% osmium tetroxide (Stevens Metallurgic Corp, New York, NY) for 1 hr. During the last step of graded acetone dehydration (25%–100%), tissue was allowed to warm (24°C) for infiltration and embedding in an Epon-Araldite resin (Electron Microscopy Sciences). Thin, 40–70-nm sections were collected on copper grids and were doubly stained by immersion in 20% uranyl acetate (Electron Microscopy Sciences) in absolute alcohol for 15 mins and then in lead citrate (12) for 10 mins.

TEM Methods to Optimize Antibody Localization. The minced pituitary gland exposed to PBS or L-692,585 was fixed in 2% paraformaldehyde and 0.02% glutaraldehyde for 2 hrs and transferred to 0.15 *M* glycine (Fisher Scientific) for 1 hr to bind free aldehyde groups. Then, tissue was dehydrated in graded methanol (25%–90%, a drop at a time; temperature was lowered stepwise from 2°C to –20°C), infiltrated, and embedded in Unicryl resin (Ted Pella, Inc., Redding, CA). Sections were cut at 40–70 nm.

Immunogold Labeling and Staining. Sections were incubated on ovalbumin for 10 mins, followed by overnight incubation at 2°C with a drop of anti-porcine GH antibody (AFP 10318545) raised in rabbit and diluted 1:800. After washing in 0.01 M PBS, sections were incubated at room temperature on a drop of gold-conjugated rabbit IgG (Sigma-Aldrich, St. Louis, MO) raised in goat and diluted 1:250 for 1 hr. Sections were stained with 2% uranyl acetate in absolute methanol for 15 mins and then with lead citrate for 40 secs. The optimal concentration for both antibodies was determined by systematically varying the concentration until minimal background was obtained. Controls, PBS substituted for the primary antibody, and incubation with only the gold-conjugated (10 nm in diameter) second antibody showed minimal background labeling. At least 200 thin sections from the control and from the stimulated pituitaries were examined at 80 kV in a JEOL JEM-100CXII electron microscope (JEOL-USA, Inc., Peabody, MA). Representative images were recorded on Kodak SO-163 electron image film (Eastman Kodak Co., Rochester, NY).

Quantitative Analysis. A total of 28 negatives were scanned (16 control and 12 stimulated), and the total number of filled, empty, and partly empty vesicles was counted independently in the positive prints by two persons. The number of vesicles in each of the three categories is listed per square micrometer of cell area.

Statistical Analysis. Means of the number of vesicles in each category per square micrometer were obtained for PBS- and L-692,585-treated groups. Data are expressed as the mean ± SEM. Simple comparisons between two groups were made by Student's t test. Data were analyzed by one-way analysis of variance, with differences considered statistically significant at $P < 0.05$.

Results

Control (resting) pituitary cells exposed to PBS contained more than twice as many filled vesicles than did the stimulated cells exposed to the GH secretagogue, L-692,585 (4.9 ± 0.21 in control, 2.3 ± 0.23 in stimulated; $P < 0.001$; Fig. 2). Stimulated cells contained nearly twice as many empty vesicles (0.6 ± 0.13 in control, 1.2 ± 0.16 in stimulated; $P < 0.05$) and two and a half times more partly empty vesicles than did control cells (1.1 ± 0.08 in control, 2.6 ± 0.12 in stimulated; $P < 0.001$; Fig. 2). However, there was no significant difference in the total number of vesicles between control and stimulated pituitary cells (Fig. 2). The remarkable increase in the number of empty and partly empty vesicles in stimulated cells compared with control cells is evident in Figure 3A and B. The intracellular distribution of empty or partially empty vesicles seemed random in stimulated cells. Immunogold labeling with GH-antibody occurs only in electron-dense GH vesicles in both control and stimulated cells (Fig. 4A and B). There was a complete absence of immunogold labeling with GH-antibody in empty vesicles or other areas of the cytoplasm. Brief exposure of cultured porcine pituitary cells to the nonpeptidyl L-692,585, ghrelin, or human GH-releasing

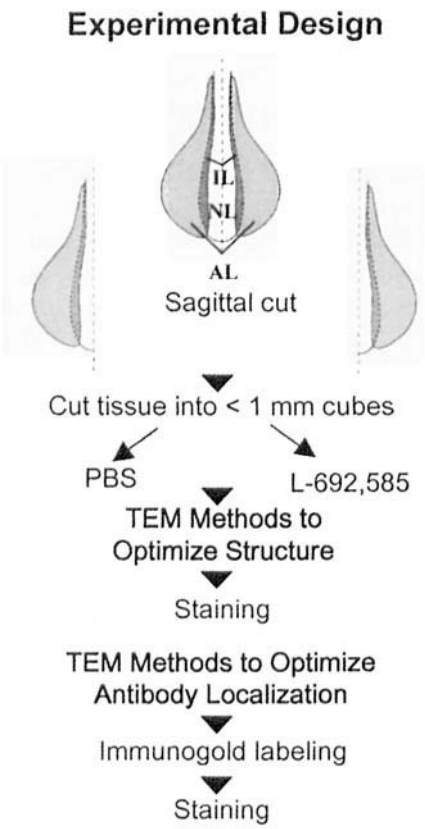

Figure 1. Description of experimental and control (resting) groups for comparing the number of secretory vesicles in growth hormone cells of 1–3-day-old porcine pituitary. AL, anterior lobe; NL, neural lobe; IL, intermediate lobe.

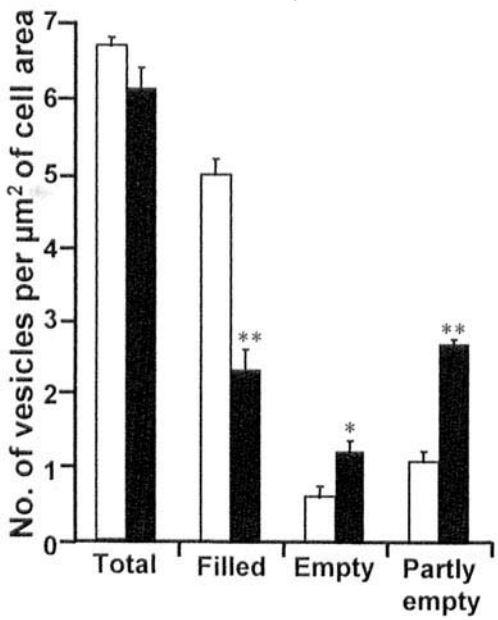

Figure 2. Total number of secretory vesicles in growth hormone cells remains unchanged in control (open bar, $n = 16$) and stimulated (solid bar, $n = 11$) cells after exocytosis. Note the changes in number of filled, empty, and partly empty vesicles between control (phosphate-buffered saline; open bar) and stimulated (L-692,585; solid bar) cells. Values are mean ± SEM. * $P < 0.05$; ** $P < 0.001$ of stimulated versus control cells.

hormone evoked a marked increase in intracellular calcium $[Ca^{2+}]_i$ concentration (13, 14). The result of stimulating live GH cells for 90 secs with L-692,585 is that filled secretory vesicles empty very rapidly, most likely by docking at the plasma membrane. After releasing vesicular contents, the empty or partly empty vesicles return to the cytoplasmic compartments of the cell.

Discussion

The classical cellular mechanism of secretion from endocrine and exocrine cells includes as the final step "total fusion" of the secretory granule with the plasma membrane (15–17). This requires the total incorporation of the secretory vesicle membrane into the cell plasma membrane. The compensatory retrieval of excess membrane by endocytosis would occur at a later time.

This final step of exocytosis has been reconsidered. In the last decade, studies clearly demonstrated that secretory vesicles transiently dock and fuse at the plasma membrane to release their vesicular contents (4–7, 10, 11, 18, 19). In addition, the discovery of a new cellular structure, designated the Porosome, has now enabled a clear understanding of secretion (4, 8, 9, 11, 20). Electrophysiological measurements, together with current TEM and atomic force microscopy studies, support this alternative model. During secretion, membrane capacitance undergoes a step increase (8, 21), which has been attributed to the secretory vesicles undergoing "transient fusion" at the plasma membrane during secretion (5–7). In slow-secreting cells like the pancreatic acinar cell in the rat, membrane capacitance involves only transient fusion events after stimulation of secretion (3). In fast-secreting cells such as nerve or mast cells, the number of secretory vesicles fusing at the plasma membrane at one time is likely much greater than in pancreatic acinar cells (1, 19). Similarly, in nerve cells, the secretory vesicles transiently fuse with the plasma membrane, leading to a step increase in plasma membrane capacitance before their dissociation from the plasma membrane (19). This would require a rapid and selective retrieval of vesicle membrane in addition to rapid fusion of synaptic vesicles at the presynaptic membrane. Rat pituitary GH cells have the ability to undergo continuous exocytosis and membrane retrieval that persists in whole-cell recording (22). Majó and colleagues (23) suggested similar secretory mechanisms for synaptic vesicles and secretory organelles in both neuronal and endocrine cells that have a highly regulated secretory pathway for intracellular communication.

Secretory vesicles that fuse with the plasma membrane in response to a physiological stimulus show similarities of synaptic proteins in both the anterior pituitary and the nerve terminal (19, 23). Although several secretory vesicle–associated proteins have been implicated in exocytosis, none are incorporated into the plasma membrane, as would occur if total fusion took place (5, 8). Studies using TEM demonstrate that following stimulation of secretion,

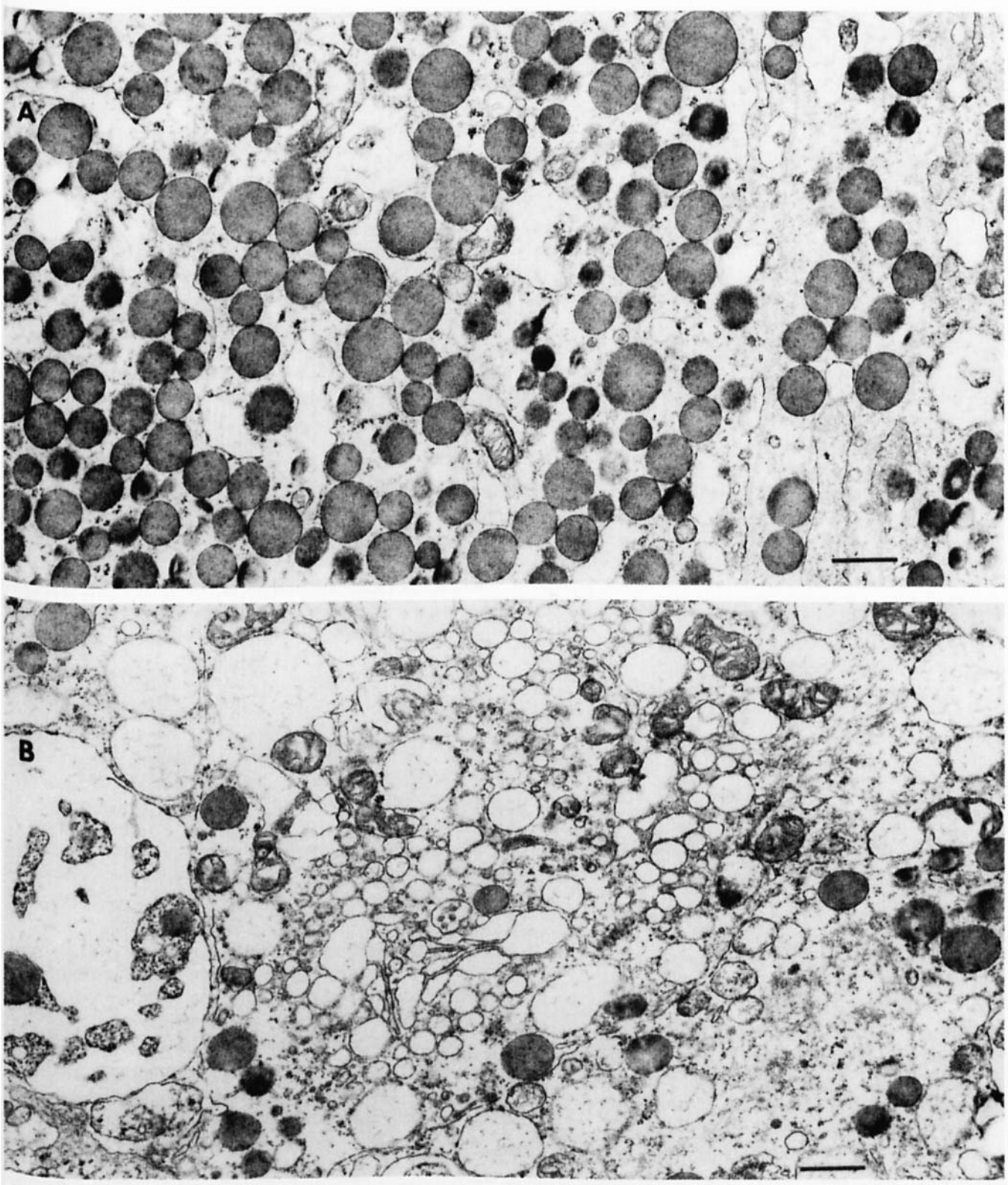

Figure 3. Transmission electron microscopy images of secretory vesicles in growth hormone cells of porcine pituitary. Note the high number of filled vesicles in control cells exposed to phosphate-buffered saline (A) and the high number of empty and partly empty vesicles in stimulated cells exposed to the secretagogue, L-692,585, for 90 secs (B). Magnification, ×22,400. Bar = 0.5 μm.

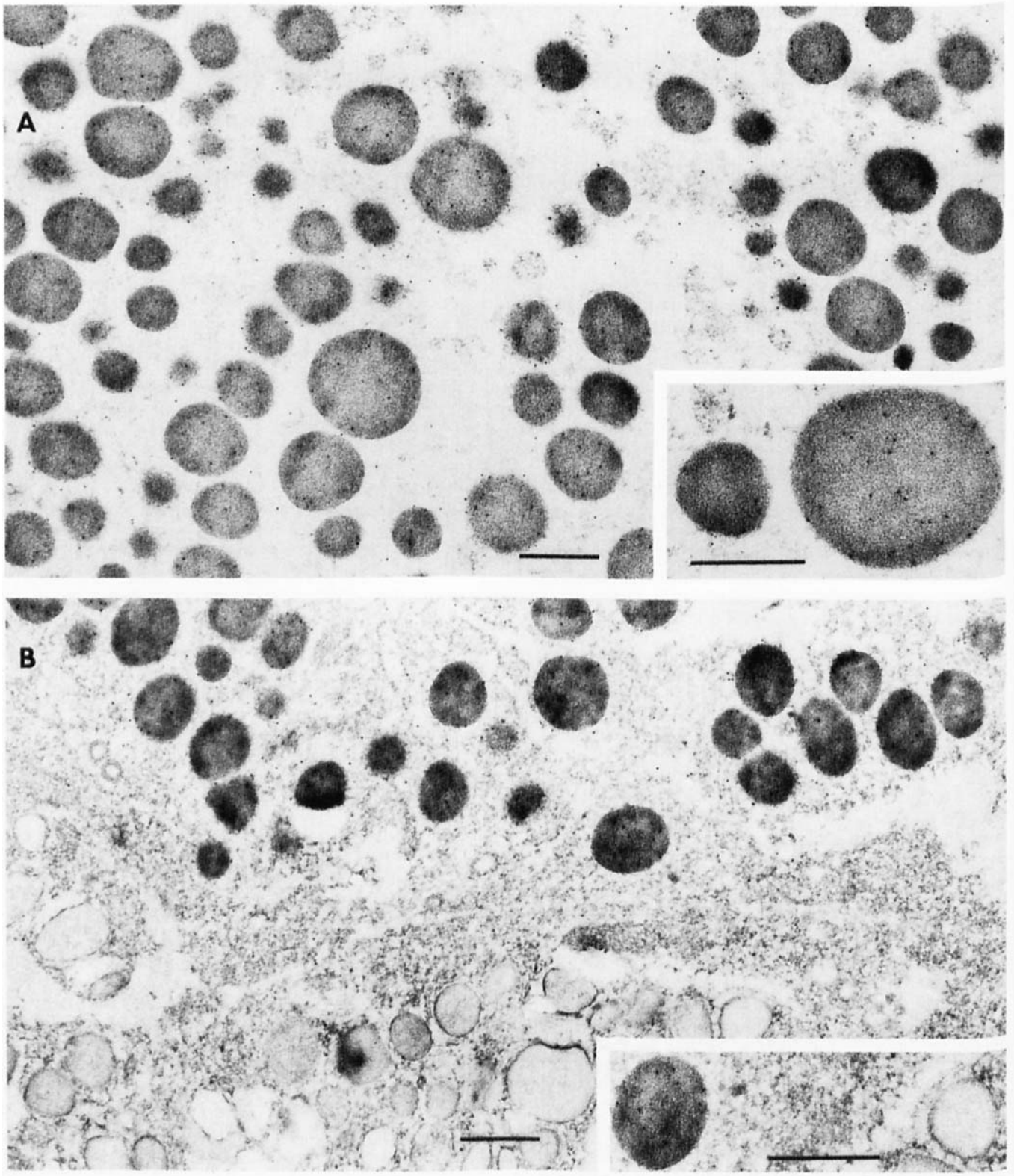

Figure 4. Transmission electron microscopy images of immunogold-labeled secretory vesicles in growth hormone (GH) cells. Note that localization of GH is only in electron-dense vesicles in both control (A) and stimulated (B) cells. After stimulation of GH secretion with the secretagogue, L-692,585, for 90 secs, there was complete absence of immunogold-labeled GH-antibody in empty vesicles (B). Magnification, ×28,000. Bar = 0.5 μm. Insets emphasize specificity of immunogold label in electron-dense vesicles of resting cells (A) and an absence of immunogold label in empty vesicles in stimulated cells (B). Magnification, ×40,000. Bar = 0.5 μm.

although there is a marked increase in the presence of empty and partly empty secretory vesicles within the cell (1, 10, 26), there is no change in the total number of secretory vesicles (2, 10, 24). These studies and other reports (3, 11, 27, 28) indicate that vesicles transiently dock and fuse to expel part of their contents during a round of vesicle docking, fusion, and secretion. Following discovery of the porosome (3, 5–9, 20) and the elucidation of the molecular mechanism of membrane fusion (4, 21) and secretory vesicle swelling (25, 26, 29), the mechanism of secretion has become clear. Following stimulation of secretion, membrane-bound secretory vesicles dock and fuse at the base of porosomes. During this time, secretory vesicles swell *via* active transport of water and ions into the vesicle lumen. This creates a buildup of pressure within the vesicles, enabling expulsion of vesicular contents. The extent of swelling dictates the amount of vesicular contents expelled. Hence, the more pressure, the more expulsion from a vesicle. Thus, the cell is able to accurately regulate secretion by regulating vesicle swelling to different degrees. Two other mechanisms regulating the expulsion of vesicular contents at the plasma membrane are the dilation of the porosome, which is actin regulated and hence requires energy, and the assembly/disassembly of SNAREs, which requires an ATPase N-ethylmaleimide-sensitive fusion protein and ATP (30). Therefore, with these two mechanisms taken together, the regulated expulsion of vesicular contents during secretion is an energy-dependent process.

From our study on GH cells, 90 secs exposure to 20 m*M* L-692,585 (GH-secretagogue) for stimulation of GH secretion was ideal for inducing rapid and effective exocytosis. L-692,585 intravenously administered to pigs also causes an immediate peak release of GH > 80 ng/ml peripheral plasma within 10 mins compared with pulsatile GH peaks of 6–10 ng/ml in placebo-treated controls (31). Intracellular signal transduction of GH-secretagogue undergoes a phosphoinositol–protein kinase C pathway that induces intracellular Ca^{2+} accumulation and depolarization, leading to exocytosis of GH-containing vesicles (13, 14). The step increase in plasma membrane capacitance of GH cells, therefore, may result from a rapid transient fusion that is consistent with the atomic force microscopy observation that shows the presence of "pits" containing "depressions" or fusion pores (porosomes) at the GH cell plasma membrane that increase markedly in diameter after L-692,585 exposure (6).

GH produced by somatotrophs within the anterior pituitary gland is secreted into sinusoids and peripheral blood during porcine fetal development. From 40 to 115 days of gestation (term, 116 days), there is a steady increase in serum GH concentration harvested from umbilical arterial blood. At day 40 of fetal development, serum levels of 3–4 ng/ml increase to 50 ng/ml by day 60, peak by day 80 at 100–115 ng/ml, and remain elevated until parturition (32). The pattern of GH secretion closely follows fetal growth and development. Postnatal changes in circulating concentrations of GH reveal an immediate decrease from birth to basal concentration of about 1 ng/ml, and peaks of 6–7 ng/ml are episodically released at 1.8/4 hrs by 21 days of age (33–35). In Chinese Meishan pigs, both circulating GH plasma concentration and pituitary GH mRNA were greatest at birth (Day 0) and decreased significantly by Days 15 and 30 (36). POU1F1 is a member of the POU-domain gene family and is a position regulator of GH, prolactin, and thyroid-stimulating hormone β in several mammalian species including the pig. POU1F1 polymorphisms were associated with growth traits, and they segregate as determined by GH mRNA and circulating GH concentrations in Chinese Meishan pigs (36). GH secretion is required for growth and nitrogen retention in this species: hypophysectomy arrests growth in young pigs, and GH treatment restores growth (37, 38). GH secretion is pulsatile in intact pigs, with episodic rhythms at approximately 3-hr intervals likely reflecting GH-mediated feedback that increases somatostatin tone at the hypothalamus and pituitary gland and attenuates GHRH and GH release (39). Hypothalamic deafferentation (40) or hypophyseal stalk transection (39) abolishes pulsatile GH release. The intravenous injection of GHRH in intact or hypophyseal stalk-transected pigs resulted in an equivalent dose-dependent peak release of GH (41). Somatotropin release-inhibiting hormone (SRIH, somatostatin) that was infused intravenously greatly decreased GHRH-induced GH secretion in hypophyseal stalk-transected and sham-operated pigs (31). Using a transorbital approach to the porcine pituitary (42), hypothalamo–hypophyseal portal blood and jugular vein blood were collected sequentially for measurement of GHRH, SRIH, and GH concentrations (43). This is the first report to reveal a pulsatile pattern of release of GHRH and SRIH in the conscious pig. The results indicated a close relationship with SRIH in GH pulse generation, but mediation of GH release cannot be explained simply by antagonism between GHRH and SRIH.

The data reported here clearly show that the total number of secretory vesicles in porcine pituitary cells is not decreased after exocytosis. Such a decrease would be required if the secretory vesicle membrane were to fuse with the plasma membrane (as in the generally accepted model for secretion). These data are consistent with a mechanism in which vesicles transiently dock and fuse at the fusion pore (porosome) to release vesicular contents after stimulation of secretion. Based on these and other supporting findings, transient fusion of secretory vesicles at the porosome on the plasma membrane may be universal in the process of exocytosis.

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