SECRETION-RELATED UPTAKE OF HORSE RADISH PEROXIDASE IN NEUROHYPOPHYSIAL AXONS

DENNISE T. THEODOSIS, JEAN JACQUES DREIFUSS, MARTIN C. HARRIS, and LELIO ORCI

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

ABSTRACT

During secretion of the neurohypophysial hormones, oxytocin and vasopressin, secretory granule membrane is added to the plasma membrane of the axon terminals. It is generally assumed that subsequent internalization of this additional membrane occurs by endocytosis. In order to study this process, we have traced the uptake of intravenously injected horseradish peroxidase by neurohypophysial axons in rats and golden hamsters.

Peroxidase reaction product within the secretory axons was found mainly in vacuolar and C-shaped structures of a size comparable with or larger than the neurosecretory granules. Our observations suggest that these large horseradish peroxidase (HRP)-impregnated vacuoles arise directly by a form of macropinocytosis. Morphometric analysis indicated that this form of membrane retrieval increased significantly after the two types of stimuli used, reversible hemorrhage and electrical stimulation of the pituitary stalk. Microvesicular uptake of HRP was found to be comparatively less.

Secretion of the neurohypophysial hormones, oxytocin and vasopressin, is now thought to occur by exocytosis. During this process, the granule membrane fuses with the plasma membrane of the axon terminal, creating an opening in the two membranes through which the hormones, together with their carrier proteins, the neurophysins, are released into the extracellular space (3, 6, 14).

If the membranes of the granules do fuse with and ultimately become incorporated into the axon plasmalemma, then persistent secretion would lead to an enlargement of the surface area of the axon endings. This enlargement does not appear to take place, so the axons must somehow retrieve and dispose of the membrane fragments added during exocytosis. Morphological evidence based on the use of extracellular markers in various tissues has shown that cells in which exocytosis occurs retrieve additional membrane by internalizing it (endocytosis) to form microvesicles and/or larger vacuolar structures.

Microvesicles are normally found in neurohypophysial nerve endings. Although they resemble the synaptic vesicles seen elsewhere in the nervous system, their function has still to be ascertained. Douglas et al. (4) have proposed that they represent the result of endocytotic membrane retrieval after neurosecretory granule release. Those authors have suggested that, after exocytosis, the granule membrane that has become incorporated at the axon surface invaginates and pinches off into the cytoplasm, in the form of coated microvesicles; these then shed their "coats" to become
smooth microvesicles (5). Supporting evidence for this hypothesis has been provided by Nagasawa et al. (17, 18), who traced the uptake of the extracellular marker, horseradish peroxidase (HRP), in neurohypophyses and reported that it was localized in microvesicles. In a similar study, however, Nordmann et al. (19) found that HRP reaction product was localized mainly in large vacuoles of a size comparable with that of the secretory granules.

Because of the differences in results presented by these previous studies, the uptake of intravenously injected HRP was traced in glands of animals stimulated to release either by electrical stimulation of the pituitary stalk or by hemorrhage. Morphometric analysis showed that, after either kind of stimulation and at all time intervals studied, peroxidase reaction product appeared within the axons mainly in vacuolar structures as large as or larger than the secretory granules. Microvesicles also contained reaction product but their number was relatively small. The study indicates that secretion-dependent endocytosis can occur by mechanisms other than micropinocytosis.

MATERIALS AND METHODS
The majority of experiments were performed on female rats (Sprague-Dawley, 250-350 g). Six male golden hamsters (80-100 g) were used for comparison in one experiment. All animals were anaesthetized with urethane (1.2 g/kg i.p.).

Peroxidase
Extracellular labeling was carried out by injecting 50-60 mg/animal of HRP obtained from Sigma Chemical Co. (St. Louis, Mo., type II) or 5-10 mg/animal of HRP obtained from Worthington Biochemical Corp. (Freehold, N. J.). Both dosages were adequate to ensure a good localization of enzyme activity. The hamsters received 20 mg/animal of HRP, Sigma Type II.

The HRP was dissolved in isotonic saline, and each animal received an intravenous injection of 1 ml of solution via the jugular vein. Animals to be stimulated were injected with the tracer 5 min before the onset of stimulation, and fixed at various time intervals thereafter. In each experiment, stimulated and unstimulated animals were given the same type and quantity of peroxidase.

Stimulation
ELECTRICAL STIMULATION: These experiments were performed on lactating rats. Bipolar stimulating electrodes were positioned onto the pituitary stalk which had been exposed via a ventral approach. Stimulation was considered adequate when it caused a rise in intramammary pressure equivalent to that induced by more than 3 mU of exogenous oxytocin (Fig. 1).

Primary Fixation and Incubation
All animals were killed by the perfusion, at room temperature, of a solution of isotonic saline followed by a 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer (pH 7.4) through the left ventricle of the heart. The time of perfusion lasted 15 min, then the neurohypophyses were quickly excised and immersed in the same fixative for a period of 2 h at room temperature. The glands were kept overnight in cold phosphate buffer and were subsequently chopped at 40 μm with a Smith-Farquhar tissue chopper.

To demonstrate peroxidase activity, the slices were treated with 3,3 diaminobenzidine and H₂O₂ according to the method of Graham and Karnovsky (9). Tissues were kept overnight in cold phosphate buffer and were subsequently chopped at 40 μm with a Smith-Farquhar tissue chopper.

FIGURE 1 Milk-ejection responses to electrical stimulation and to exogenous oxytocin. Records show intramammary pressure changes to intravenous oxytocin (A, D, E, G), to intravenous HRP (C), and to electrical stimulation of the pituitary stalk (B, F). The responses are consecutive, but the record is not continuous, except between F and G. In B, a 5-s train of stimuli (2 ms duration, biphasic, rectangular) was delivered at 50 Hz; this yielded a response approximately equivalent to that following the injection of 1.5 mU oxytocin (A). HRP injection produced no detectable change in intramammary pressure (C) and did not significantly alter the responses to exogenous oxytocin (D, 1.5 mU; E, 0.5 mU). In F, a stronger stimulus was applied to the pituitary stalk (10 s, 0.8 mA, 50 Hz). This produced a polyphasic response approximately equivalent to that produced by 5 mU oxytocin (G). Fixation was started after the end of record G. Vertical and horizontal calibrations are the same for all records.
from animals that had received only saline injections were treated similarly.

**Preparation for Electron Microscopy**

The 40-μm slices were postfixed in 2% OsO₄ in Millonig’s buffer for 60–90 min at room temperature. After a brief rinse in buffer, the tissues were immersed for 30 min, in the dark, in a solution of 1.5% uranyl acetate dissolved in 50% ethanol. Tissues were then dehydrated in a graded series of ethanol and embedded in Epon (16).

Ultrathin sections (60–80 nm thick) were cut with a diamond knife on an LKB 3 ultramicrotome. These were mounted on naked grids and stained with lead citrate and/or uranyl acetate. The grids were examined with a Philips 300 electron microscope.

**Morphometry**

Morphometric analysis was carried out on neurohypophysial glands fixed at the following periods: 1 min and 5 min after electrical stimulation of the pituitary stalk; 5 min and 4 h after the onset of hemorrhage. Glands from unstimulated rats that had been exposed to HRP for equivalent periods were studied at the same time. Ultrathin sections from randomly selected blocks (three rats/group, three blocks/rat) were examined and photographed (10 micrographs/section).

The electron micrographs were recorded on 70-mm film at an initial magnification of 11,000. A carbon-grating replica with 2,160 lines/mm was recorded on each film for calibration. The films were examined in a table projector unit fitted with a double lattice test system (9:1 surface ratio).

The relative volume density of all peroxidase-containing structures larger than 150 nm was determined by the point counting method (26, 27). All the cross points of coarse lattice lines lying on axon cytoplasm were first counted to estimate the area of cytoplasm present in the sample ($P_B$). Then the number of cross points of fine lattice lines lying on the peroxidase-impregnated structures were recorded ($P_f$). The relative volume of these structures ($V_e$) was then calculated from the equation:

$$V_e = \frac{P_f}{9P_B}.$$

The number of microvesicles containing peroxidase reaction product and present in the same micrographs in which the larger structures were tabulated was also counted. Their mean diameter having been derived, their numerical density was calculated according to the formula:

$$N_v = \frac{N_a}{d + T} = \frac{N_f}{P_eL^3(d + T)}$$

where $N_v$ = number of microvesicles per unit volume; $N_a$ = number of microvesicles per unit section area; $N_f$ = number of profiles counted; $d$ = mean measured diameter of the microvesicles; $L$ = spacing of points on test lattice; $T$ = section thickness, 60 nm.

**RESULTS**

**Short-Term Exposure to HRP**

Neurohypophysial axons and axon endings are readily recognized with the electron microscope because of their numerous secretory granules. These granules, 150–200 nm in diameter, were characterized by an electron-dense core that was separated from the granule limiting membrane by a pale submembranous area. They were found distributed among microtubules, mitochondria, and many electron-lucent microvesicles.

Up to 15 min after the injection of peroxidase, variable amounts of reaction product were seen within the axons as well as in the adjacent perivascular space in both unstimulated and stimulated preparations (Figs. 2 and 3). Within the axons, reaction product was found mainly in vacuoles and C-shaped structures (mean diameter, 200 nm). By C-shaped structures are meant curved cisternae enclosing a cytoplasmic space often devoid of any electron density. Associated with the C-figures were smaller vesicles and tubules, with or without reaction product. The amount of reaction product apparent within the vacuoles and C-shaped structures varied, and the black deposits were usually found closely associated with the vacuolar membrane. C-figures entirely empty of any reaction product were present as well. Impregnated microvesicles were visible and, in rare instances, coated microvesicles also contained the tracer (see Fig. 6). In glands fixed 1–5 min after stimulation, peroxidase reaction product was also localized within large indentations of the axons (Figs. 5 and 6), and in tubular structures (Fig. 5) presumably invaginating from the axon plasmalemma. Large vacuoles and C-shaped structures were identified in unstimulated and stimulated glands of animals that had received no peroxidase (Fig. 4).

**Long-Term Exposure to HRP**

Neurohypophyses from rats exposed to peroxidase for 30, 60, 120, and 240 min after reversible hemorrhage were examined. In all these groups, HRP reaction product was visible mainly in large vacuoles and C-shaped figures. In addition, peroxidase became apparent in a number of multivesicular bodies (Fig. 7). These were membrane-
bounded vacuoles containing a variable number of smaller vesicles. In the multivesicular bodies, peroxidase reaction product was seen both within the small vesicles and in the space between the vesicles and the membrane of the containing vacuole. Similar multivesicular bodies, as well as vacuoles, labeled with peroxidase were found within cell bodies of the supraoptic nucleus 4 h after the injection of the protein.

Golden Hamsters

For comparison, neurohypophyses from six golden hamsters stimulated by hemorrhage were examined. At both 2 min and 1 h after hemorrhage, the predominant peroxidase-containing structures were large vacuoles and C-shaped structures comparable to those seen in the axon terminals of the rat (Figs. 8 and 9). Reaction product
FIGURE 4 Electron micrograph of a neurohypophysial axon from a rat not exposed to HRP and fixed 1 min after electrical stimulation. C-shaped structures are indicated by arrows. A cluster of microvesicles is shown at mv. × 62,000.

was apparent in multivesicular bodies 1 h after stimulation.

**Morphometric Analysis**

The comparative distribution of HRP-containing vacuoles and C-figures in axon cytoplasm was ascertained by determining the relative volume (basic symbol of stereology expressed as numbers without units) they occupied at various time intervals after the two types of stimulation (Fig. 10). In the unstimulated glands, this relative volume amounted to approximately $10^{-3}$ and did not vary significantly with time. A highly significant increase ($P < 0.001$, Student's $t$ test) was apparent both 1 and 5 min after electrical stimulation. A comparable increase occurred at both 5 and 240 min after hemorrhage. Moreover, there was no significant difference in the relative volume occupied by the vacuolar structures at 5 and 240 min after the onset of hemorrhage.

The number of HRP-containing microvesicles present in the same axon cytoplasm in which the relative volume of vacuoles and C-figures was determined was ascertained by calculating their numerical density (Fig. 11). Their numbers did not differ significantly in the unstimulated groups. A highly significant increase did occur 1 min after electrical stimulation. However, no increase was evident after hemorrhage. In considering these differences, it must be noted that, in contrast to the mean relative volumes shown in Fig. 10, the data representing mean numerical densities show a much higher coefficient of variation.

It is not possible to directly compare the results shown in Figs. 10 and 11, since they are expressed in different units. In order to make such a comparison, the numerical densities of microvesicles were multiplied by the mean volume occupied by each microvesicle (i.e. $N_v \cdot (d^3)/6$ where $d = 58$ nm, the mean microvesicle diameter). This yielded an estimate of the relative volume occupied by the impregnated microvesicles which could then be directly compared to the relative volume occupied by the vacuoles and C-figures.

Since the data for each group (controls, electrical stimulation and hemorrhage) at the different time intervals studied did not differ markedly, they were pooled and averaged. The mean relative volumes after each type of stimulation were then expressed as the difference from that of the control group. The results of these computations are shown in Table I. The values in the third column, which give the percentage of the total volume uptake due to microvesicular uptake, indicate that the latter accounts for only 0.2–1.0%.

For a given volume, the total membrane area internalized will be larger if uptake occurs in small vesicles rather than in large ones. Since the surface to volume ratio for spherical structures is inversely proportional to their diameter, and since the mean diameter of the vacuoles and C-shaped structures is approximately 3.5 times that of the microvesicles, it follows that the relative volume occupied by the impregnated microvesicles ought to be mul-

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1 The peroxidase-containing vesicles showed an average diameter of $58 \pm 15$ SD ($n = 240$). The microvesicles found within neurohypophysial axons probably represent more than one population since clustered, unimpregnated microvesicles had an average diameter of $35 \pm 9$ SD ($n = 1960$), significantly lower than that of dispersed, unlabeled microvesicles, $52 \pm 13$ SD ($n = 1910$).
FIGURES 5 and 6  Electron micrographs of neurohypophyses from rats injected with Worthington HRP.

Figure 5  Electron micrograph of neurohypophysial terminals 5 min after the onset of hemorrhage. Circular, HRP-containing structures appear to be invaginating at the cell surface (arrows). Note the tubular structure (T) containing reaction product. × 53,000.

Figure 6  Electron micrograph of a neurohypophysial terminal from a rat fixed 2 min after electrical stimulation. The dense reaction product within the extracellular space (E) delimits a large invagination of the axon plasmalemma. Microvesicles, both coated (arrow) and uncoated (dotted arrow), are seen to contain reaction product. × 54,000.

Figure 7  Electron micrograph of axon terminals from a rat killed four hours after reversible hemorrhage. Peroxidase reaction product is still seen within vacuoles (V) and C-shaped structures (C) but also in a number of multivesicular bodies (mvb). C-shaped structures with no reaction product are also apparent (arrows). M, mitochondrion. × 68,000.

dultiplied by 3.5 to yield an estimate of the relative amount of membrane area taken into the cell’s interior by microvesicles. Even with this correction, at least 95% of the internalized membrane fragments after secretion surrounded vacuoles and C-shaped structures and, at most, 5% of the endocytosed membrane was microvesicular.

DISCUSSION

This study has shown that after hormone release by rat and hamster neurohypophyses, peroxidase reaction product rapidly appeared within the axon terminals, mainly enclosed in large smooth-surfaced vacuoles and C-shaped structures. Microvesicles were also marked with peroxidase but mor-
FIGURES 8 and 9 Electron micrographs of axon terminals from hamster neurohypophyses. The animals had received Sigma (Type II) HRP.

FIGURE 8 Low-power electron micrograph of tissue fixed 2 min after the onset of hemorrhage. Peroxidase reaction product is seen within the extracellular space (E) while in the terminals it is found in a large number of vacuoles. Only a small number of microvesicles contains peroxidase (arrows). × 23,000.

FIGURE 9 Neurohypophysial terminals from a hamster killed 1 h after the onset of hemorrhage. Large vacuoles contain reaction product while impregnated microvesicles are also apparent. The majority of the microvesicles, however, are free of reaction product. × 48,000.

Phometric analysis indicated that they accounted for only 1% of total volume uptake in the unstimulated glands and for less than 1% of the total volume uptake associated with stimulation.

These results are at variance with those of Naganawa and co-workers (17, 18) who first traced the uptake of HRP in stimulated neurohypophyses. We have no ready explanation for these differences since we did carry out a number of experiments using, as they did, a much larger concentration (90 mg/animal) and a different type of HRP (Sigma type VI) and fixed the neural lobes by immersion. We found that the neurohypophysial axons contained endocytotic figures similar to those described and identified at length in the present paper.

According to the concept of exocytosis followed by micropinocytosis (3), each exocytotic event involving one neurosecretory granule would require the formation of some 10-25 microvesicles if the cell surface is not to expand. Both our data and those of previous investigators present little quantitative evidence to indicate such an increase after hormone secretion.

No increase in the relative volume occupied by microvesicles was detected by Reinhardt et al. in rat neurohypophyses after 5 days of dehydration (21). On the other hand, Livingston (15) did report an increase in the number of microvesicles per nerve ending after saline drinking. This increase occurred in nerve endings abutting on the perivascular space and was accompanied by a corresponding decrease in microvesicular density in the axoplasm remote from the neurovascular contact area, suggesting that hormone release may produce a redistribution rather than an increase in...

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FIGURE 10 Mean values (± SEM) of the relative volumes (x 10^-3) occupied by HRP-impregnated vacuoles and C-shaped structures in unstimulated and stimulated neurohypophyses. All animals had been exposed to HRP 5 min before stimulation. P values were obtained by Student's t test.

FIGURE 11 Mean values (± SEM) of the numerical densities (x 10^-2/μm^3) of HRP-impregnated microvesicles found in the same unstimulated and stimulated preparations from which the data of Fig. 10 were obtained.

TABLE 1

| Time Following | Electrical | Control | Hemorrhage |
|----------------|------------|---------|------------|
|                | Stimulus   |         |            |
| 1 min          | 0.27 ± 0.03| 0.10 ± 0.02| 0.09 ± 0.02|
| 5 min          | 0.41 ± 0.04| 0.11 ± 0.03| 0.09 ± 0.03|
| 240 min        | 0.09 ± 0.02| 0.11 ± 0.02| 0.49 ± 0.05|

Volume Uptake by HRP-Containing Microvesicles, Vacuoles, and C-Shaped Structures in Control Neurohypophyses and in Neurohypophyses Stimulated by Electrical Stimulation or Hemorrhage

|                  | V_v | V_w | V_v/V_w |
|------------------|-----|-----|---------|
| Control          | 0.99 x 10^-6 | 1.03 x 10^-6 | 1.0    |
| Electrical      | 3.30 x 10^-3 | 2.24 x 10^-3 | 0.7    |
| Hemorrhage*     | 4.25 x 10^-3 | 1.06 x 10^-3 | 0.2    |

V_v, relative volume occupied by impregnated vacuoles and C-figures.
V_w, relative volume occupied by impregnated microvesicles.
* Values given express additional volume uptake.
† Values express the percentage of total volume uptake due to microvesicles.

Since we do not know the rates of uptake of the extracellular marker nor whether the internalized membranes are interconverted one to the other within the axon cytoplasm, we cannot exclude the possibility that uptake by microvesicles, followed by rapid, and thus, undetectable transport to vacuoles has taken place. However, the large invaginations and tubular formations that were marked with peroxidase and were seen within 1 min after stimulation suggest that the large vacuoles may arise by a form of macropinocytosis or tubular invagination of the axon plasmalemma.

In summary, the results of the present investigation suggest that neurosecretory axon endings are capable of endocytosis via processes other than micropinocytosis. Macropinocytosis is also taking place but contributes relatively little to total HRP uptake.

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uptake and thus seems unlikely to be the principal means of excess membrane retrieval after exocytosis. The ultimate fate of the membrane fragments internalized after secretion is still being investigated.

We wish to thank Dr. A. Perrelet for his assistance in the preparation of the manuscript and Mrs. M. Sidler-Ansermet for her excellent photographic work.

This research was supported by the Fonds National Suisse de la Recherche Scientifique (grants 3.553.75 and 3.257.74).

Received for publication 8 December 1975, and in revised form 2 April 1976.

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