Morphometric analysis of stimulus-induced changes in the distribution of secretory granules in a subclone of PC12 cells: a rapid freeze-substitution study

SHINJI MATSUTANI¹, HISAKO KOJIMA², YOICHI TAMAI² and NOBORU YAMAMOTO¹

¹Department of Functional Morphology, School of Nursing, and ²Department of Biochemistry, School of Medicine, Kitasato University, Kitasato, Sagamihara, Kanagawa 228, Japan

ABSTRACT

Stimulus-induced changes in the distribution of secretory granules were analyzed in MR31 cells, a subclone of PC12 cells, with the aid of rapid freeze-substitution electron microscopy. The technique enabled us to detect the short-term change as early as 10 sec after the onset of depolarizing stimulus. Morphometric analysis revealed that secretory granules located in the cell periphery moved outward in response to the stimulus, and that the movement attenuated within 5 min, taking account of both the temporal changes in the distribution of secretory granules and the time course of [³H]dopamine release from MR31 cells.

Secretion of neurotransmitters and hormones is controlled by various factors, and the intracellular mechanisms underlying the phenomenon have been intensively studied. In this kind of research, the morphological approach is essential, because secretion apparently involves ultrastructural changes such as the movement of secretory granules and exocytosis, and these changes may reflect the operating mechanisms. While the intracellular movement of secretory granules following secretory stimuli may be one of the regulatory steps, the morphological details of this process are not fully understood, since it is difficult to trace movements which may terminate soon after stimulation stops. For this purpose, a rapid freeze-substitution electron microscopy seems suitable. The technique greatly reduces the artifacts inherent in conventional chemical fixation (6, 14), and enables us to capture the short-term changes in fine structures.

Here, we showed temporal changes in the distribution of secretory granules during the secretory response in a subclone of PC12 cells transfected with the *ras* oncogene (designated MR31 cells, see reference 8). Electron microscopic specimens were prepared by a rapid freeze-substitution technique from the cells cultured on domestic wrapping film, the procedure which has advantages for processing

of specimens (11). In addition, stimulus-induced dopamine release from MR31 cells was measured in relation to the morphological study.

MR31 cells were grown in RPMI 1640 (Gibco) containing 5% fetal calf serum, 10% horse serum, 100 units/ml of penicillin and 100 μ g/ml of streptomycin. For the morphological experiments, cells were plated on collagen-coated domestic wrapping film, Saran Wrap (Asahi Chemical Industry; see reference 11), at about 3×10^4 cells/cm². Two days after plating, the cells were washed with balanced salt solution (BSS, 150 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 10 mM glucose, 0.1 mM EDTA, 0.5 mM ascorbic acid, 0.3 mM pargyline hydrochloride and 25 mM HEPES, pH 7.4), and the Saran Wrap with attached cells was cut into small pieces. Then the cells were stimulated with BSS with an elevated K⁺ concentration (60 mM KCl with a substitution of NaCl). This solution is referred to as high-K⁺ BSS. Next the sample was mounted on the plunger of a rapid freezing device (Eiko, RF-2). For short-term stimulation, the samples were first mounted on the plunger and then stimulated with a drop of high-K⁺ BSS. They were slammed against a copper plate cooled with liquid nitrogen, and quickly transferred into 2% OsO₄ in acetone precooled in a mix-

Fig. 1 Electron-micrographs of a rapid freeze-substituted MR31 cells. A: A non-stimulated cell. B: A cell stimulated for 1 min. The number of secretory granules close to the plasma membrane is larger in the stimulated cell. The nucleus is seen in B (N). Bar, 500 nm

ture of dry-ice and acetone, and stored at -80°C for 2 days. After the substitution, the cells together with the Saran Wrap were embedded in epoxy resin. As previously reported (11), Saran Wrap was easily cut, trimmed and thin-sectioned with resin. The samples were stained with uranyl acetate and lead tartrate, and examined under a Hitachi H-7000 electron microscope.

To estimate the distribution of secretory granules, cells including a nuclear profile were randomly photographed, and the distance between the plasma membrane and the central point of each secretory granule was measured on the photographs, which were printed at a final magnification of $\times 25,000$. The secretory granules of MR31 cells were easily identified at this magnification by their appearance: an electron-dense core and a limiting membrane as in chromaffin and PC12 cells (Fig. 1). Data were collected from duplicate experiments. In order to reduce the possible sampling bias caused by the variety of cell shapes, only secretory granules located within 400 nm of the plasma membrane were taken into account. The depth distribution was calculated as a ratio of the number of secretory granules located within 200 nm of the plasma membrane to that located within 400 nm. The proportion of the number of secretory granules located within 400 nm of the plasma membrane to the total number of secretory granules appearing in cell profiles remained almost unchanged during the stimulation period ($36.5 \pm 0.9\%$).

To measure dopamine release, cells were plated into 24-well collagen-coated culture plates (Corning) at a density of 3×10^4 cells/cm² and cultured for 2 days. They were then washed with BSS, and incubated with [³H]dopamine ($0.125 \mu\text{Ci}/\text{well}$, Du Pont-New England Nuclear) in BSS for 1 h at 37°C . Then the cells were washed 4 times with BSS at intervals of 10 min, and stimulated with high- K^+ BSS. The incubation medium was collected from each well and radioactivity was counted. The radioactivity remaining in the cells was measured after their solubilization with 0.5 N NaOH. The released [³H]dopamine was expressed as a percentage of the total [³H]dopamine in the cells prior to stimulation, and was designated as % release. Release activity (% release/min) was calculated from the difference between two values at successive time points.

The time course of high- K^+ -evoked dopamine release from MR31 cells seemed to consist of two components (Fig. 2): an initial transient release, which was most prominent at 30 sec after the onset of stimulation, and a slow sustained secretion, which decayed gradually and ran down within 5 min.

A rough electron microscopical survey showed that there was no drastic change in the distribution of secretory granules during the stimulation period. As in resting cells, they were seen in the cytoplasm and about one third of them was located within 400 nm of the plasma membrane (Fig. 1). However, a close examination revealed that the dis-

Fig. 2 Time course of [^3H]dopamine release from MR31 cells. Each value represents the mean $\pm$ SEM of 4 experiments. Error bars smaller than symbols are omitted.

tribution of secretory granules in the periphery of the cell changed in response to stimulation (Table 1). The ratio of secretory granules located close to the plasma membrane (less than 200 nm apart) increased as early as 10 sec after stimulus onset, transiently returned to the control level at 30 sec, when the release activity was highest (Fig. 2), then increased again. After 5 and 10 min, the ratios slightly declined toward that of resting cells, since the ratios at 5 and 10 min do not statistically differ from that of control cells ($P > 0.05$, t -test).

It can be assumed that the temporal changes shown in Table 1 reflect the balance between the outward movement and release of secretory granules. Taking into account the time course of secretory response, these results suggest that some secretory granules were driven toward the cell periphery upon stimulation, and that the movement ran down within 5 min, since both the release activity and change in the ratio could be hardly detected after 5 min. A transient decrease in the ratio at 30 sec may reflect the increased release of superficially located secretory granules which exceeds the outward movement of more deeply situated granules. On the other hand, the increase in the ratio at 1 min may result from a reduced secretory activity (Fig. 2) and a persistent movement of granules.

The rapid freeze-substitution technique has advantages for the analysis of ultrastructural changes which may occur and terminate within a short period of time. As shown in Fig. 2, stimulus-induced dopamine release from a population of

Table 1 Stimulus-induced Changes in the Distribution of Secretory Granules Located in the Cell Periphery

Stimulus duration	Cell number	Secretory granules	
		0-400 nm ^a	0-200 nm/0-400 nm ^b
0 sec	35	240	0.429
10	31	218	0.523 ^c
20	21	208	0.548 ^d
30	24	193	0.482
1 min	30	279	0.516 ^c
5	22	221	0.507
10	25	257	0.510

^aNumber of secretory granules located within 400 nm of the plasma membrane. ^bRatio of the number of secretory granules located within 200 nm of the plasma membrane to that located within 400 nm. ^cSignificantly higher than in controls (0 sec) at $P < 0.05$ (t -test). ^dSignificantly higher than in controls at $P < 0.02$.

MR31 cells rises up to 30 sec and decays within several minutes. Accordingly, it must be difficult to detect the accurate images of exciting cells, especially in the initial rising phase, through the conventional chemical fixation which proceeds much slowly to mask actual changes occurring in the cell. Thus, the rapid freeze-substitution technique seems essential for the ultrastructural studies on secretion.

The present study has revealed that depolarizing stimuli cause the outward movement of secretory granules as well as release. Taking the time course of secretory response into account, the movement may be initiated within 10 sec of the stimulus onset and terminate within 5 min. Observations of living chromaffin cells have demonstrated that secretory granules show continuous fluctuations and gliding or saltatory movements (3, 16), and that stimulus-induced exocytosis run down without a significant decrease in population of secretory granules located close to the plasma membrane, due to the fluctuation. Our present result has also shown that the stimulus-induced change in the proportion of secretory granules close to the plasma membrane is relatively small, although it is statistically significant (Table 1). Taken together, the stimulus-induced outward movement of secretory granules which can be statistically detected may reflect a decrease in the irregularity of the fluctuation.

The stimulus-induced movement of secretory granules demonstrated in the present study may serve as a supply of readily releasable or available

secretory granules. The regulation of availability for release has been investigated in terms of cytoskeleton-binding proteins. In adrenal chromaffin cells, an actin-binding protein, fodrin (1, 12, 13), and an actin-severing protein, gelsolin (2, 15), are thought to regulate availability, while the involvement of a phosphoprotein, synapsin I, has been reported in synaptic terminals (5, 7, 9, 10), although the latter has not been detected in chromaffin cells. Since the actions of these proteins are directly or indirectly controlled by calcium, the stimulus-induced movement of secretory granules observed in the present study, which may also be triggered by calcium, can be initiated by their action. In other words, their action might be a prerequisite for movement. To date, the localization of fodrin and gelsolin in chromaffin cells has been demonstrated mainly on a light microscopic level (12, 15). Their ultrastructural localization (4) and stimulus-induced changes must be elucidated for a better understanding of stimulus-secretion coupling.

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