DIFFERENT MODES OF SINGLE GRANULE FUSION EVENTS IN NEUROENDOCRINE CELLS AT REST

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Introduction. It is thought that the release of hormones from neuroendocrine cells and neurotransmitters from nerve terminals requires vesicle delivery to the plasma membrane sites, where vesicles dock and get primed for exocytosis, which occurs upon a stimulus. Lactotrophs, cells of the anterior pituitary, release hormone prolactin from secretory granules through a fusion pore that is formed upon the fusion between the granule membrane and the plasma membrane. Previously it was shown that spontaneous hormone discharge from a single vesicle is slower than the release under stimulation due to the kinetic constrains of the fusion pore openings (1). However, slow spontaneous hormone release may also be a consequence of a relatively narrow fusion pore. In the present work, we tested the hypothesis that docked hormone-containing vesicles at rest are engaged in exocytosis yielding fusion pores of sub-nanometer diameters, too small for discharge of relatively large peptide hormone prolactin (23 x 10^3 g/mol, diameter = ~5.2 nm).

Methods. We monitored the permeation of fluorescent dye FM 4-64 and HEPES molecules through spontaneously forming fusion pores of prolactin containing vesicle by time-lapse confocal microscopy in resting lactotrophs, expressing synaptopHluorin (spH), a pH sensitive green fluorescence protein fused to the luminal side of the vesicle-membrane protein synaptobrevin-2 (2). When expressed in exocytotic vesicles, spH enables optical discrimination between the unfused (faint or no fluorescence signal) and fused vesicles (greatly increased green fluorescence signal). The experiment were performed in saline solution containing 10 mM or 100 mM HEPES. Simultaneously with the spH fluorescence changes, we monitored the loading of vesicles by extracellularly added red fluorescent dye FM 4-64, which stains the vesicle matrix upon the opening of the fusion pore (1,3).

Results. Exocytosis of prolactin-containing vesicles (n = 36) in saline solution containing 10 mM HEPES resulted in a rapid increase in spH fluorescence, indicating that in these vesicles spontaneous fusion with the plasma membrane exposed spH to the external alkaline medium via the fusion pore. Based on the capability of vesicles to load the FM 4-64 dye we distinguished two populations of spontaneous fusion events, type-I (Figure A) and type-II events (Figure B). Type-I events were devoid of FM 4-64 loading (n= 19), on the contrary in type-II events vesicles loaded the FM 4-64 (n = 17). In both type of events, following the peak in spH fluorescence intensity signal, the signal either decayed with exponential function (transient events, 64% events, n = 23), implying reacidification of the vesicle lumen due to the resealing of the fusion pore, or it remained at an elevated level during the monitoring (persistent events, 36% events; n = 13), indicating that in these events the vesicular lumen remained in contact with the extracellular space through an open fusion pore. The absence of vesicle entry by FM 4-64 dye molecules (608 g/mol, diameter = ~0.9 nm) in type-I exocytic events and the absence of time-course modulation of these events in the presence of increased concentration of HEPES molecules (100 mM HEPES; data not shown), which are 2.5-fold smaller (238 g/mol, diameter = ~0.5 nm) than FM 4-64, suggest that both FM 4-64 and HEPES molecules were unable to pass
the fusion pore in the type-I events. In contrast, the fusion pore diameter in type-II events appears to be wider allowing the passage of molecules as large as 0.9 nm diameter, but perhaps also several times larger peptide hormones such as prolactin (1).

**Figure.** Spontaneous exocytotic events of type-I (A) and type-II (B). A sequence of images representing a single vesicle (upper panel) and the time-dependent fluorescence intensity changes at the vesicle site (lower panel), separately for spH (green) and FM 4-64 (red). Numbers on the plots indicate the time points at which the upper images were recorded. The arrows show the vesicles undergoing fusion. Scale bar: 1 μm.

**Conclusions.** The results show that one half of spontaneous exocytotic events exhibited fusion pore openings associated with a change in spH fluorescence, but were unpermeable to FM 4-64 and HEPES molecules, indicating an open fusion pore diameter of <0.5 nm, much smaller than the molecular size of secretory peptides in these vesicles. It appears that at rest more than half of the exocytotic vesicles undergo fusion yielding sub-nanometer fusion pore diameters, precluding discharge of the peptide hormones. In addition to forming a barrier for hormone release, we propose that the presence of such unproductive exocytosis at rest may contribute to the stability of the anchoring of vesicle to the exocytotic site.

**Literature:**