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Vesicle size determines unitary exocytic properties and their sensitivity to sphingosine

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ABSTRACT

Neuroendocrine cells contain small and large vesicles, but the functional significance of vesicle diameter is unclear. We studied unitary exocytic events of prolactin-containing vesicles in lactotrophs by monitoring discrete steps in membrane capacitance. In the presence of sphingosine, which recruits VAMP2 for SNARE complex formation, the frequency of transient and full fusion events increased. Vesicles with larger diameters proceeded to full fusion, but smaller vesicles remained entrapped in transient exocytosis. The diameter of vesicle dense cores released by full fusion exocytosis into the extracellular space was larger than the diameter of the remaining intracellular vesicles beneath the plasma membrane. Labeling with prolactin- and VAMP2-antibodies revealed a correlation between the diameters of colocalized prolactin- and VAMP2-positive structures. It is proposed that sphingosine-mediated facilitation of regulated exocytosis is not only related to the number of SNARE complexes per vesicle but also depends on the vesicle size, which may determine the transition between transient and full fusion exocytosis.
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1. Introduction

Nerve cells and some endocrine cells contain 2 types of secretory vesicles differing mainly in their size: small synaptic-like vesicles and large dense core vesicles (LDCVs) (Klyachko and Jackson, 2002). The functional significance of this morphological difference is unclear. Here, we studied unitary exocytic events of peptidergic vesicles and asked whether their properties are altered as a function of vesicle diameter.

Prolactin (PRL), an anterior pituitary hormone (Riddle et al., 1933), is stored in LDCVs with a diameter of 100–500 nm (Angleson et al., 1999; Jorgačevski et al., 2011; Zorec et al., 1991a; Smets et al., 1987). Pituitary lactotrophs release PRL by regulated exocytosis (Zorec et al., 1991b; Katz, 1969), which involves the fusion of the vesicle membrane with the plasma membrane. An important step in the release of PRL is the formation and the subsequent expansion of a fusion pore which connects the vesicle lumen with the cell exterior (Spruce et al., 1990; Breckenridge and Almers, 1987; Vardjan et al., 2007). In the limiting case of fusion pore expansion, its diameter widens to such an extent that the vesicle membrane gets fully incorporated into the plasma membrane (full fusion exocytosis) (Vardjan et al., 2007; Jena, 1997; Heuser and Reese, 1973). However, some fusion pores can reversibly close after being open for several hundreds of milliseconds or even several seconds, which may hinder discharge of the vesicle cargo (Stenovec et al., 2004) (transient or kiss-and-run exocytosis (Ceccarelli et al., 1973; Alvarez de Toledo et al., 1993)) and it is the predominant type of exocytosis in pituitary lactotrophs (Vardjan et al., 2007; Jorgačevski et al., 2008).

Sphingosine has been shown to facilitate regulated exocytosis by recruiting vesicle-associated membrane protein 2 (VAMP2; also known as synaptobrevin 2) for the formation of the soluble *n*-ethylmaleimide-sensitive fusion factor attachment protein receptor (SNARE) complex (Darios et al., 2009). In addition, it can be generated on the outer leaflet of the plasma membrane, followed by subsequent internalization across the plasma membrane into the cell

Abbreviations: LDCVs, large dense core vesicles; PRL, prolactin; VAMP2, vesicle-associated membrane protein 2; SNARE, soluble *n*-ethylmaleimide-sensitive fusion factor attachment protein receptor; C_m, membrane capacitance; SIM, structured illumination microscopy; G_p, pore conductance; Im, imaginary and real (Re) parts of the admittance signal; FWHM, full width at half maximum; VOCCs, voltage activated calcium channels.

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(Tani et al., 2005; Hannun and Obeid, 2008). However, how sphingosine modulates the properties of unitary exocytic events is not known.

Here, we asked whether sphingosine affects the properties of unitary exocytic events. We monitored changes in membrane capacitance (C_m), a parameter linearly related to the area of the plasma membrane (Neher and Marty, 1982). The results show that the addition of sphingosine increases the frequency of transient and full fusion exocytic events dose dependently. However, sphingosine differentially affected vesicles of different sizes. Full fusion was more frequent within the fraction of vesicles with a larger diameter, whereas transient fusion was confined to the vesicles with relatively smaller diameter. These results were further confirmed by super-resolution optical microscopy, where we measured the diameter of immunolabeled PRL-containing vesicles beneath the plasma membrane and the diameter of nondegradable vesicle dense cores on the outer leaflet. In addition, these studies revealed a positive correlation between the diameter of PRL- and VAMP2-positive structures. These results show that sphingosine modulation of regulated exocytosis not only depends on SNARE molecules, but also on vesicle size and fusion pore stability.

2. Materials and methods

2.1. Cell preparation

Lactotroph-enriched cell cultures were isolated from anterior pituitaries of adult male Wistar rats (Ben-Tabou et al., 1994; Rituper et al., 2013). Lactotrophs were obtained by enzymatic dispersion, enriched with Percoll gradient (the purification yielded cultures with >90% purity, data not shown), cultivated on glass coverslips coated with poly-L-lysine and maintained in DMEM (D5546, Sigma, USA), supplemented with 10% newborn calf serum and 2 mM L-glutamine in an atmosphere of humidified air (95%) and CO_2 (5%). All experiments were performed at room temperature within 4 days of cell isolation. The animals were cared for in accordance with the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences and the Directive on Conditions for Issue of License for Animal Experiments for Scientific Research Purposes (Official Gazette of the Republic of Slovenia 43/07). The animal use procedures were approved by the Veterinary Administration of the Republic of Slovenia (Apr. No. 3440-29/2006 and 34401-29/2009/2).

2.2. Solutions

Extracellular solution (ECS) contained (in mM): NaCl 130, KCl 5, $CaCl_2$ 8, $MgCl_2$ 1, D-glucose 10, HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] 10, and pH 7.2/NaOH. Osmolarity was measured with a freezing-point osmometer (Osmomat 030; Gonotech, Germany). The solution used for stimulation was prepared from ECS with the addition of 100 μM or 200 μM sphingosine (D-(+)-erythro-1,3-dihydroxy-2-amino-4-*trans*-octadecene) (dissolved in 14 M DMSO). To exclude the possible effects of DMSO (vehicle), we also tested ECS containing an equal concentration of DMSO as the solution for stimulation.

2.3. Electrophysiology

We measured changes in C_m using the compensated cell-attached technique with a dual-phase lock-in patch-clamp amplifier (sine wave frequency (f), 1591 Hz; 111 mV root mean square; SWAM IIC; Celica, Slovenia) as already described (Rituper et al., 2013; Kabaso et al., 2013). All recordings were performed with fire-polished, thick-wall pipettes coated with silicone elastomer Sylgard (Down Corning, USA) and a low surface level of bath solution (200 μl per coverslip) to reduce the noise level. Pipettes, which were prepared daily, had resistance in the range of 2–5 MΩ. The pipette potential was held at 0 mV. The phase of the lock-in amplifier was adjusted to nullify the changes in the real part of the admittance signal (Re) in response to 10 fF calibration steps in the imaginary part of the admittance signal (Im), reporting changes in C_m. We first recorded changes in C_m under spontaneous conditions for ~350 s and then added the sphingosine solution in the ratio (v/v) of 1:1, with the final concentration of sphingosine reaching 50 or 100 μM, and recorded changes in C_m for an additional ~350 s. For the control, we used the vehicle solution in a ratio (v/v) of 1:1. Transient events in the Im signal that exhibited measurable projections to the Re signal (crosstalk) were used to calculate fusion pore conductance [$G_p = (Re^2 + Im^2)/Re$] (Lollike and Lindau, 1999) and vesicle capacitance [$C_v = (Re^2 + Im^2)/Im]/\omega$, where ω denotes the angular frequency. The fusion pore diameter was estimated by using the equation $G_p = (\pi \times r^2)/(\rho \times \lambda)$, where r denotes the fusion pore radius, ρ is the estimated resistivity of saline (100 Ω cm), and λ is the estimated fusion pore length (15 nm) (Spruce et al., 1990; Vardjan et al., 2007; Hanna et al., 2009). Vesicle diameter was determined by assuming spherical geometry of vesicles and by using specific membrane capacitance (C_m = 9 fF/μm^2). Cells were observed under Zeiss Axioobserver A1 (Germany).

2.4. Imunnocytochemistry

Lactotrophs were washed once with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (dissolved in PBS) for 15 min, and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Nonspecific background staining was reduced by a blocking buffer, containing 3% bovine serum albumin (BSA) and 10% goat serum in PBS (37 °C, 1 h). Lactotrophs were incubated sequentially with primary antibodies, diluted in 3% BSA in PBS, and incubated at 37 °C for 2 h or overnight at 4 °C. Cells were then rinsed four times in PBS, incubated with secondary antibodies at 37 °C for 45 min and fixed on glass slides using SlowFade Gold antifade reagent (Life Technologies, USA). We used the following antibodies: polyclonal rabbit anti-PRL (1:60; Millipore, USA), monoclonal mouse anti-VAMP2 (1:1000, Synaptic Systems, Germany) in combination with the secondary antibodies against rabbit and mouse IgG conjugated to fluorescent dyes (Alexa Fluor® 546 or Alexa Fluor® 488, both 1:600, Life Technologies, USA). For Vybrant DID cell-labeling, lactotrophs were incubated in 50 μM sphingosine (or in vehicle solution) for 6 min at 37 °C and then washed. Plasma membrane was labeled with 25 μM Vybrant DID cell-labeling solution (Invitrogen, Germany) for 4 min at 37 °C, and cells were fixed in 2% paraformaldehyde (dissolved in PBS) for 10 min. The labeling protocol was then the same as described above, but without the 0.1% Triton X-100 permeabilization. We used the following antibodies: monoclonal mouse anti-PRL (1:300; Thermo Scientific, Germany) in combination with the secondary antibodies against mouse IgG conjugated to fluorescent dyes (Alexa Fluor® 488, 1:600, Life Technologies, USA). We used confocal microscop Zeiss LSM 510 and oil immersion objective (Zeiss, Plan-neofluar 63×, NA 1.4) and structural illumination microscopy (SIM) Zeiss Elyra PS1 with oil immersion objective Zeiss, 63×, NA 1.4).

2.5. Data analysis

Comparison of all the parameters in electrophysiological recordings under spontaneous conditions and after the addition of vehicle solution revealed no significant differences ($P > 0.05$).

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