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SPHINGOMYELIN DERIVATIVES INCREASE THE FREQUENCY OF MICROVESICLE AND GRANULE FUSION IN CHROMAFFIN CELLS

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Abstract—Sphingomyelin derivatives like sphingosine have been shown to enhance secretion in a variety of systems, including neuroendocrine and neuronal cells. By studying the mechanisms underlying this effect, we demonstrate here that sphingomyelin rafts co-localize strongly with synaptosomal-associated protein of 25 Kda (SNAP-25) clusters in cultured bovine chromaffin cells and that they appear to be linked in a dynamic manner. In functional terms, when cultured rat chromaffin cells are treated with sphingomyelinase (SMase), producing sphingomyelin derivatives, the secretion elicited by repetitive depolarizations is enhanced. This increase was independent of cell size and it was significant 15 min after initiating stimulation. Interestingly, by evaluating the membrane capacitance we found that the events in control untreated cells corresponded to two populations of microvesicles and granules, and the fusion of both these populations is clearly enhanced after treatment with SMase. Furthermore, SMase does not increase the size of chromaffin granules. Together, these results strongly suggest that SNARE-mediated exocytosis is enhanced by the generation of SMase derivatives, reflecting an increase in the frequency of fusion of both microvesicles and chromaffin granules rather than an increase in the size of these vesicles.

Key words: exocytosis, neurotransmission, chromaffin cells, sphingomyelin, cell capacitance.

INTRODUCTION

The fusion of vesicles with the plasma membrane is a key process in the exocytotic release of neurotransmitters and hormones by distinct cell types. Exocytosis appears to be mediated by members of the SNARE family of proteins, which govern membrane trafficking in cells (Weber et al., 1998). However, lipids are also essential regulators of exocytosis (Zimmerberg et al., 1991; Davletov et al., 2007; Lang et al., 2008) and cone-shaped lipids favor the appropriate membrane curvature for the fusion of secretory vesicles (Chernomordik and Kozlov, 2003). In addition to facilitating an appropriate membrane structure for vesicle fusion, lipids could also directly influence the secretory machinery by binding to individual SNAREs or to the fusion complex, formed by SNAREs associating with other elements. A good example of this type of regulation is that of arachidonic acid (AA), a lipid released from phospholipid membranes by phospholipases that upregulates syntaxin-1 and increases the incorporation of this protein into fusogenic SNARE complexes (Rickman and Davletov, 2005; Connell et al., 2007). Sphingosine is another signaling lipid that is released from the backbone of sphingolipids by sphingomyelinase (SMase) activity. Sphingosine interacts with vesicular synaptobrevin II, promoting the formation of the ternary complex and facilitating vesicle exocytosis in neuronal and endocrine systems (Darios et al., 2009). Thus, these signaling lipids clearly affect different SNARE proteins and they serve to increase the number of ternary complexes, thereby enhancing the secretory properties of neuroendocrine cells (Darios et al., 2009, 2010).

Although these molecular interactions appear to be well characterized, the steps of the secretory pathway that could be affected by these lipids are less evident. In this sense, it was recently suggested that in addition to modifying the overall secretory profile, these signaling lipids could alter the extent and kinetics of individual amperometry spikes in chromaffin cells (Wightman et al., 1991; Garcia-Martinez et al., 2013), or the proportion of transient vs. full fusion events in lactotrophs (Flasker et al., 2013). Hence, these lipids also appear to regulate the final fusion step itself.

In the present work, we have used confocal microscopy and capacitance techniques to further study the ability of sphingomyelin derivatives to modulate exocytosis at the global and the individual vesicle level. Our results show that these signaling lipids enhance secretion by increasing the frequency of exocytotic

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Abbreviations: AA, arachidonic acid; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MSD, Mean Square Displacement; SMase, sphingomyelinase; SNAP-25, synaptosomal-associated protein of 25 Kda.

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events and by promoting the fusion of microvesicles and chromaffin granules.

EXPERIMENTAL PROCEDURES

Chromaffin cell preparation and culture

Chromaffin cells were isolated from bovine adrenal glands by collagenase digestion, and they were further separated from the debris and erythrocytes by centrifugation on Percoll gradients as described elsewhere (Gutierrez et al., 1988; Giner et al., 2007). The cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 10 μM cytosine arabinoside, 10 μM 5-fluoro-2′-deoxyuridine, 50 IU/ml penicillin and 50 μg/ml streptomycin. The cells were then seeded at a density of 150,000 cells/cm^2 in 35-mm Petri dishes (Costar, Corning, NY, USA), and they were used between the third and sixth days *in vitro*.

For patch-clamp experiments chromaffin cells from 3 to 6-month-old rats were isolated using a protocol very similar to that used for bovine chromaffin cells. Briefly, the adrenal medulla was dissociated for 30 min at 37 °C with collagenase (3 mg/ml, Type IA: Sigma C-9891) in the presence bovine serum albumin (BSA, 10 mg/ml: Sigma A-7906, Sigma, Milano, Italy) and in a dissociation buffer containing (in mM): NaCl 137, KCl 5, HEPES 25, glucose 10 (osmolarity 300 mOsm/kg, pH 7.2). The cells were suspended in DMEM supplemented with 10% FCS, 50 IU/ml penicillin, and 50 μg/ml streptomycin. The cells were plated on 35-mm dishes coated with poly-L-lysine (Sigma) and maintained at 37 °C in a water-saturated atmosphere containing 5% CO_2 and 95% O2. Experiments were performed at room temperature on cells maintained for 1–4 days in culture. These experiments were conformed to University of Seville and International Guidelines on the ethical use of animals and efforts has been made to minimise the number of animals used and their suffering.

Generation of synaptosomal-associated protein of 25 Kda (SNAP-25) GFP construct and cell transfection

The cDNA corresponding to the SNAP-25a isoform (Bark and Wilson, 1994) was cloned in-frame into the XhoI and BamHI sites of the pEGFP-C3 expression vector (Clontech, Palo Alto, CA, USA) that encodes a red-shifted variant of wild-type GFP (Cormack et al., 1996) in order to express a SNAP-25a protein fused to EGFP at the C-terminus (construct GFP-SNAP-25, Gil et al., 2002). The DNA construct carried an internal HindIII site close to the 5′-end and the aforementioned BamHI site at the 3′-end, sites that were used to substitute the SNAP-25 sequence in the original vector with the modified GFP-SNAP-25. Chromaffin cells were transfected using the Amaxa basic nucleofector kit for primary mammalian neuronal cells according to the manufacturer's instructions (Program O-005, Amaxa GmbH, Koehl, Germany). In some experiments chromaffin cells expressing GFP-SNAP-25 were incubated with a fluorescent ceramide analog (BODIPY-Texas-Red ceramide). This lipid was transformed into the corresponding fluorescent derivative of sphingomyelin, which will release free sphingosine in the proximity of SNAREs, using an experimental protocol involving the sequential incubation of fluorescent ceramide (5 μM) for 30 min at 4 °C followed by a 2-h incubation at 37 °C in medium lacking serum (Tyteca et al., 2010).

Confocal microscopy

Fluorescent confocal images were obtained with an Olympus Fluoview FV300 confocal laser system mounted on an IX-71 inverted microscope and incorporating a 100X PLAN-Apo oil-immersion objective (1.45 n.a.). Excitation was achieved using Ar (488 nm wavelength) and HeNe (543 nm wavelength) visible light lasers, and images were processed using the ImageJ program for centroid determination, ROI measurements, and image average as described in previous works (Neco et al., 2004; Lopez et al., 2007). Additional, ImageJ plug-ins have been used for particle centroid tracking (Multitracker plug-in), and co-localization analysis (colocalization plug-in).

Electron microscopy

Bovine chromaffin cell pellets were fixed for 2 h at 4 °C with 2.5% glutaraldehyde in 0.2 M cacodylate buffer [pH 7.0] and the cells were then washed with a solution of 0.2 M cacodylate buffer, sucrose and distilled water overnight. After post-fixing with 1% osmium tetroxide in 0.2 M cacodylate buffer for 2 h and extensive washing, the cells were stained with 2% aqueous uranyl acetate for 2 h. The pellets were then washed again, dehydrated through an ethanol series (30%, 50%, 70%, 80%, 96% and 99%: 15 min each) and treated with propylene oxide for 30 min at room temperature. Finally, the cells were embedded in epoxy resin, and ultra-thin sections (70 nm) were obtained using a Leica UC6 ultramicrotome and transferred to copper grids (200 mesh). After staining with uranyl acetate for 5 min and lead citrate for 1 min, the ultrathin sections were analyzed on a JEOL 1011 a 80-kv transmission electron microscope with Gatan BioScam mod 792 digital camera to capture the images. Chromaffin granule size was determined using Image J software with plug-ins to automatically determine the particle area.

Cell membrane capacitance measurements

The whole-cell or on-cell configurations of the patch-clamp technique were used to assay exocytosis from chromaffin granules by measuring the cell membrane capacitance. In whole-cell experiments, we measured the ionic currents (calcium, sodium and potassium) evoked by depolarizing pulses from the membrane potential and immediately after the application of the pulse, we measured the cell membrane capacitance with the "sine + dc" method (Lindau and Neher, 1988) implemented in the lock-in extension software of an EPC-10 patch-clamp amplifier (HEKA, Germany). This