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Review

The action of sphingomyelinase in lipid monolayers as revealed by microscopic image analysis

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ABSTRACT

In recent years, new evidence in biomembrane research brought about a holistic, supramolecular view on membrane-mediated signal transduction. The consequences of sphingomyelinase (SMase)-driven formation of ceramide (Cer) at the membrane interface involves reorganization of the lateral membrane structure of lipids and proteins from the nm to the μm level. In this review, we present recent insights about mechanisms and features of the SMase-mediated formation of Cer-enriched domains in model membranes, which have been elucidated through a combination of microscopic techniques with advanced image processing algorithms. This approach extracts subtle morphological and pattern information beyond the visual perception: since domain patterns are the consequences of subjacent biophysical properties, a reliable quantitative description of the supramolecular structure of the membrane domains yields a direct readout of biophysical properties which are difficult to determine otherwise. Most of the information about SMase action on simple lipid interfaces has arisen from monolayer studies, but the correspondence to lipid bilayer systems will also be discussed. Furthermore, the structural changes induced by sphingomyelinase action are not fully explained just by the presence of ceramide but by out-of equilibrium surface dynamics forcing the lipid domains to adopt transient supramolecular pattern with explicit interaction potentials. This rearrangement responds to a few basic physical properties like lipid mixing/demixing kinetics, electrostatic repulsion and line tension. The possible implications of such transient codes for signal transduction are discussed for SMase controlled action on lipid interfaces.

Contents

Abbreviations: SMase, Sphingomyelinase; PLA_2, phospholipase A_2; Cer, N-acylsphingosine; SM, sphingomyelin; chol, cholesterol; GUVs, giant unilamellar vesicles; ROIs, Regions of Interest; hgd, Highest Gap Distance; GGVF, generalized gradient vector flow; LE, liquid-expanded; LC, liquid-condensed; *ld*, liquid-disordered; *lo*, liquid-ordered; P^2/A, perimeter^2/area; AFM, Atomic Force Microscopy

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1. Introduction

The varied effects of the so-called "signaling lipid mediators" on the membrane structural dynamics have profound influences on the activity of lipolytic enzymes acting on membranes. This is a key aspect of molecular information exchange that represents a linking point between the local molecular events of metabolism (open to biochemical pathways) and the structural membrane dynamics (related to most functions in cell biology) [1–3]. Lipid metabolism is characterized by the fact that most substrates and products are water insoluble. Lipid substrate organization in supramolecular aggregates is essential for lipolytic enzyme efficiency and constitutes a key point in enzyme kinetics regulation [4]. As summarized in a recent review [5] phospholipase kinetics have at least four inter-dependent levels of regulation, namely: a) on the initial adsorption/partition or relocation of the enzyme in the interface; b) on the enzyme precatalytic activation that frequently determines the length of the lag period for activity; c) on the expression of catalytic activity itself through the reaction rate and d) on the extent of product formation. In addition, membrane biophysical properties undergo changes as a consequence of phospholipase action [4,6,7].

An important group of phospholipases called sphingomyelinases (SMases) hydrolyze the membrane constituent sphingomyelin (SM) to water-soluble phosphocholine and to water-insoluble ceramide (Cer). Cer is an important second messenger with unusual biophysical features such as a high tendency to segregate in liquid-condensed, Cer-enriched domains [8,9]. Cer shows strong interaction with SM and scarce miscibility with other cell membrane components [10–12]. SMase-driven aggregation of Cer-enriched domains beyond the μm scale has been identified in monolayers [13], giant liposomes (GUVs) [8], and as so-called membrane platforms in Jurkat T Cells [14]. Lateral Cer organization itself can alter physical membrane parameters like permeability [15], or introduce membrane bending that sculptures 3D membrane vesicles or apoptotic bodies [16,17]. Finally, the structural membrane reorganization itself can couple back to cellular signaling events through clustering of receptors, recruitment of intracellular signaling molecules, or exclusion of inhibitory signaling factors in Cer-enriched domains that have been reviewed in [14,18]. In summary, it has been recognized that a cellular amplifier mechanism leads from molecular activation (SMase-driven SM→Cer conversion) to self aggregated Cer-enriched domains at a supramolecular level, and structural reorganization of membrane pattern on a 2D and 3D level, all of which alter biophysical membrane parameters and couples back on receptor recruitment with the respective signal transduction down to molecular levels.

While it is becoming increasingly clear that Cer exerts a fundamental part of its physiological effects through changes in the physical properties of the membrane [19–21], details about how the kinetics of the SM→Cer conversion itself mediates morphological and pattern organization of the Cer-enriched domains have been studied by real-time monolayer approaches during the last decade [13,22,23]. As we describe in this review, the study of SMase action on lipid monolayer interfaces with Langmuir troughs coupled to quantitative time lapse fluorescence microscopy using image processing techniques has opened the access towards a more integrated view on the surprising plurality of structural and functional properties of a single enzymatic reaction.

In this context, the physiological consequences of the enzymatic production of Cer might follow similar mechanism for known members of the SMase family [6]. For availability reasons, most of the biophysical studies of SMase action reported in this review were done using bacterial enzymes. In vivo generation of Cer should be addressed in a case specific manner.

2. Enzymatic generation and visualization of Cer-enriched domains: a monolayer approach

Direct visual evidence in real time for SMase-induced formation of Cer-enriched domains was first provided by our group [13]. For this work lipid monomolecular films (monolayers) were used. Lipid monolayers provide a powerful model to study lipid–lipid and lipid–protein interactions since the thermodynamics of this system is accessible to the researcher keeping simultaneous control of molecular parameters like molecular packing, surface pressure and compressibility [24]. In addition, the Langmuir trough can be coupled to the stage of a microscope allowing the study of laterally segregated phases or domains [5,25]. When applied to the study of phosphohydrolytic reactions taking place on the membrane, this technique is unique in allowing the continuous monitoring of both the enzyme activity and the surface pattern while keeping control of organization parameters (Fig. 1). Taking advantage of the difference in cross-sectional mean molecular area occupied by SM and Cer (84 $Å^2$ and 51 $Å^2$ respectively at a surface pressure of 10 mN/m, see Fig. 1b) the time course for the SMase-driven enzymatic conversion of SM→Cer in lipid monolayers can be determined by recording the reduction of the surface area necessary to maintain a constant surface pressure (Fig. 1c and [26]). The monolayer trough allows simultaneous visualization of the monolayer by epifluorescence microscopy (Fig. 1d). Using a fluorescent probe that preferably partitions to the liquid-expanded phase, the evolution of the monolayer and domain formation can be followed in real time [13].

The use of fluorescent probes which are added to the system in study has to be handled with care, since molecular impurities, even at very low concentrations, can alter lipid mixing features like domain shape, phases extent, or even promote phase separation under specific conditions. Pioneer work by Mohwald on lipid monolayers considered the influence of impurities, like fluorescent probes on lipid domain growth [27]. More recently, a careful study by Perez-Gil's laboratory shows that the addition of 1 mol% of NBD-PC on DPPC films reduces the extent of the LC phase area up to 20% in conditions when the probe progressively accumulates in a defined area [28]. In both studies the effect is conceived as inefficiency in the phase demixing process induced by the presence of impurities. Furthermore, fluorescent probe perturbation of lipid phase segregation was also reported in bilayers. Keller's group demonstrated that the addition of as little as 0.2 mol% of $DiIC_{12}$ to DOPC/DPPC/cholesterol (chol) GUVs rises 6 °C the mixture miscibility transition temperature [29,30]. Most of these crescent evidences have been developed by means of comparison with probe-free imaging techniques. On this regards Force Microscopy, Brewster Angle Microscopy and Imaging Elipsometry have become excellent choices for the exploration of probe-free lipid monolayers or flat supported bilayers [31].

Additional consideration should be made with the extensive exposure of lipid membranes to oxidizing agents. Enlargement of liquid-ordered domains in chol/SM/phospholipid mixtures has been reported by means of the production of oxidized lipid products driven by the exposure of lipid monolayers to air [32] or by photo-oxidation in GUVs [33]. Excitation of the fluorophore in the presence of oxygen may result in peroxidation of the unsaturated bonds of membrane lipids. It was proposed that oxidized products may act either by