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Revisiting transbilayer distribution of lipids in the plasma membrane

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ABSTRACT

Whereas asymmetric transbilayer lipid distribution in the plasma membrane is well recognized, methods to examine the precise localization of lipids are limited. In this review, we critically evaluate the methods that are applied to study transbilayer asymmetry of lipids, summarizing the factors that influence the measurement. Although none of the present methods is perfect, the current application of immunoelectron microscopy-based technique provides a new picture of lipid asymmetry. Next, we summarize the transbilayer distribution of individual lipid in both erythrocytes and nucleated cells. Finally we discuss the concept of the interbilayer communication of lipids.

1. Introduction

The organization of lipids in the plasma membrane is determined by biosynthesis, incorporation of exogenous lipids, intracellular lipid traffic, and lipid remodeling in the plasma membrane. Transbilayer lipid asymmetry was first proposed in 1972, the same year that the "fluid mosaic model" was proposed by Singer and Nicolson (Singer and Nicolson, 1972). Using a membrane-impermeable amino-reactive reagent, Gordesky reported the enrichment of phosphatidylethanolamine and phosphatidylserine in the inner leaflet of human erythrocyte membranes (Gordesky et al., 1972). Bretscher also showed asymmetric distribution of phosphatidylethanolamine in red blood cell membranes (Bretscher, 1972b). These two approaches were applicable only to amino-phospholipids such as phosphatidylethanolamine and phosphatidylserine. Soon after, Verkleij et al. introduced phospholipase treatment to analyze the transbilayer distribution of other lipids such as phosphatidylcholine and sphingomyelin (Verkleij et al., 1973b). Robert Bittman is one of the first who examined the transbilayer distribution of cholesterol (Bittman and Rottem, 1976; Blau and Bittman, 1978).

Today, the concept of "lipid asymmetry" is well accepted. However, most of the available data on lipid asymmetry originated from the distribution of the main phospholipids in red blood cells, which have only a single membrane. Studying the transbilayer distribution of lipids in multi-membrane systems such as nucleated mammalian cells is still a technical challenge. In addition, the transbilayer distribution of cholesterol is still a matter of debate.

The recent development of proteins and peptides that bind specific lipids enabled us to visualize individual lipids on the outer and inner leaflets of the plasma membrane under electron microscopy. This technique allows us to revisit lipid asymmetry. In this review we critically explain the methods of examining the transbilayer distribution of lipids. There is no perfect protocol, and thus a complementary approach is required to obtain satisfactory results. We then describe the update of transbilayer distribution of individual lipids in the plasma membrane.

2. Methods to examine transbilayer distribution of lipids

2.1. Factors that influence the measurement of transbilayer distribution of lipids

Although several techniques are reported to study transbilayer distribution of lipids, none of them is a perfect method. Following factors have to be considered in choosing and applying methods.

(1) Scrambling of lipid bilayer during treatment

To study transbilayer lipid distribution, it is essential that scrambling of lipids between outer and inner leaflet does not occur during treatment. Production of non-bilayer lipids such as ceramide and diacylglycerol may alter the bilayer structure. For example, ceramide can induce transbilayer lipid movement through a lamellar-to-non-lamellar phase transition (Contreras et al., 2003). Lysophospholipids also modify membrane structure.

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Long time incubation of intact cells may induce re-organization of lipid bilayer.

(2) Labeling selectivity

The ideal method is to detect lipids without labeling. However, for the moment, it is technically difficult to examine the lipid composition of each lipid bilayer leaflet by mass spectrometry. The second choice is to identify lipids by either biochemical or histochemical method. Non-specific modifications such as enzyme treatment need further identification procedure such as thin-layer chromatography or mass spectrometry. Recent advances in the development of lipid-specific proteins and peptides enable us to distinguish and visualize different head groups of lipids. These probes selectively label specific lipids simply by incubating with the specimen. The lipid specificity of these probes has to be extensively characterized both *in vivo* and *in vitro* before use. Antibodies that recognize phosphatidylcholine with specific fatty acid composition have also been reported (Kuge et al., 2014).

(3) Labeling efficiency

Labeling efficiency is determined by the dissociation constant (Kd) and the size of the probes. The Kd of lipid-binding probes may be affected by the lipid density as well as the membrane environment around the lipids. As observed for lipid-binding probes, the sensitivity of phospholipases is affected by the lipid density and lipid composition of the membrane. Thus, appropriate control experiments are always necessary to evaluate the results in cell experiments.

(4) Endogenous vs exogenous lipids

Exogenously added spin-labeled or fluorescence-labeled lipid analogs (Devaux et al., 2002) as well as "clickable" lipid precursor (Iyoshi et al., 2014) have been employed to examine transbilayer distribution of phospholipids. Fluorescent- and spin-labeled lipid analogs provided an important concept of transbilayer lipid movement. These lipid analogs also revealed the fate of the lipids after incorporation to the plasma membrane. However, because lipids are small (molecular weight ~1,000), subtle alterations of the molecule may affect the transbilayer distribution of lipids. In addition, it is extremely difficult to prove that exogenous lipids are in equilibrium with endogenous lipids. Thus it is important to compare the obtained results with those of endogenous lipids.

2.2. Biochemical methods

Lipid asymmetry was first revealed by biochemical technique using red blood cell membranes. This technique is still a strong approach to study lipid asymmetry. Biochemical methods are composed of two independent procedures: (1) Selective irreversible modification of outer leaflet lipids either by conjugation of chemicals or by enzymatic degradation. Alternatively, outer leaflet lipids are selectively exchanged using lipid transfer protein. (2) Analysis of the ratio of modified/unmodified lipids of the samples.

Water-soluble, membrane-impermeable amino-reactive reagents such as 2,4,6-trinitrobenzen sulfonic acid (TNBS) or *N*-Hydroxysuccinimide (NHS) esters of biotin (EZ-Link Sulfo-NHS-Biotin) (Clark et al., 2013) have been employed to examine the transbilayer distribution of phosphatidylethanolamine and phosphatidylserine (Kobayashi and Pagano, 1989; Sleight and Pagano, 1985). In addition to amino-reactive reagents, lactoperoxidase-mediated radioiodination has been employed to examine lipid asymmetry in *Acholeplasma laidlawii* (Gross and Rottem, 1979). Chemical labeling of lipids requires 10–60 min. Chemical labeling has not been applied to relatively inert phosphatidylcholine or sphingomyelin.

Selective hydrolysis of outer leaflet lipids by a variety of exogenous phospholipases has been widely employed to examine the transbilayer lipid distribution of major lipids in the plasma membrane. Phospholipids are extracted after enzyme treatments and analyzed by thin-layer chromatography. Complete degradation of outer leaflet lipids require 10–120 min, depending on cell types and experimental conditions. A potential problem of this technique is that the reaction products (lysophospholipid, ceramide, diacylglycerol) are membrane active and thus may reorganize membrane bilayer during treatment.

The use of purified lipid transfer proteins that exchange lipids from the outer leaflet of donor membranes to acceptor liposomes has also been employed to study transbilayer lipid distribution. This technique requires longer incubation time (1–6 h).

The methods listed in this section are, in principle, best for single-membrane system, such as erythrocytes and gram-positive bacteria. Application of these methods to multi-membrane systems such as nucleated cells requires additional measures to obtain highly purified membrane preparations and the restriction in the redistribution of lipids from membrane to membrane.

2.3. Histochemical methods

The transbilayer distribution of several fluorescent lipid analogs in the plasma membrane has been reported (Hale and Schroeder, 1982; McIntyre and Sleight, 1991; Mondal et al., 2009). In these experiments, fluorescent lipids located at the outer leaflet were extinguished using appropriate quenchers. Measurement of fluorescence before and after quenching either by microscopy or spectroscopy provided quantitative results on the transbilayer distribution of fluorescent lipids. Recently, choline analog, propargylcholine (Jao et al., 2009) was employed to examine transbilayer distribution of choline-containing phosphatidylcholine and sphingomyelin (Iyoshi et al., 2014). In this experiment, cells were grown in the presence of propargylcholine. Propargylcholine was selectively incorporated into phosphatidylcholine and sphingomyelin. Propargylcholine-containing lipids were then biotinylated by "click" reaction using biotin-azide. Next, biotin-labeled lipids were observed by electron microscopy after staining with anti-biotin antibodies and protein A-golds. The lipid analogs often give a high signal compared to endogenous lipids. However, as described above, it is important to compare the obtained results with those of endogenous lipids.

The recent development of various proteins and peptides that bind specific lipids made it possible to selectively label endogenous lipids. Conjugation of these proteins and peptides with green fluorescent protein (GFP) or fluorescent dye provides lipid-specific probes that are applicable to label lipids in living cells. These protein probes for lipids are either added to the medium to label outer leaflet lipids of the plasma membrane or expressed in the cytoplasm to label lipids located to the cytoplasmic leaflet of the membrane. Labeling cells from outside requires 5–30 min, whereas the expression of GFP-probes takes 12–24 h. Probes have to be extensively characterized, and experimental conditions must be carefully controlled. The followings are pitfalls of utilizing protein probes for lipids: (1) multivalent proteins (cholera toxin B subunit, IgM etc) can crosslink lipids (Hammond et al., 2005; Jung et al., 2009; Lingwood et al., 2008); (2) the recognition of lipids by the specific probes may be affected by how the lipids are distributed in the membrane (Makino et al., 2015); (3) the target lipid may already be bound or masked by other proteins, which inhibit the binding of probes; (4) overexpression of the probes in cytoplasm may alter the lipid distribution; (5) since proteins are much bigger