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Imaging approaches for analysis of cholesterol distribution and dynamics in the plasma membrane

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ABSTRACT

Cholesterol is an important lipid component of the plasma membrane (PM) of mammalian cells, where it is involved in control of many physiological processes, such as endocytosis, cell migration, cell signalling and surface ruffling. In an attempt to explain these functions of cholesterol, several models have been put forward about cholesterol's lateral and transbilayer organization in the PM. In this article, we review imaging techniques developed over the last two decades for assessing the distribution and dynamics of cholesterol in the PM of mammalian cells. Particular focus is on fluorescence techniques to study the lateral and inter-leaflet distribution of suitable cholesterol analogues in the PM of living cells. We describe also several methods for determining lateral cholesterol dynamics in the PM including fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS), single particle tracking (SPT) and spot variation FCS coupled to stimulated emission depletion (STED) microscopy. For proper interpretation of such measurements, we provide some background in probe photophysics and diffusion phenomena occurring in cell membranes. In particular, we show the equivalence of the reaction-diffusion approach, as used in FRAP and FCS, and continuous time random walk (CTRW) models, as often invoked in SPT studies. We also discuss mass spectrometry (MS) based imaging of cholesterol in the PM of fixed cells and compare this method with fluorescence imaging of sterols. We conclude that evidence from many experimental techniques converges towards a model of a homogeneous distribution of cholesterol with largely free and unhindered diffusion in both leaflets of the PM.

1. Introduction

1.1. Current models of plasma membrane architecture

The plasma membrane (PM) of living cells is a highly specialized structure that transmits and controls the information flow between a cell and its environment. It also creates a permeability barrier against ions and macromolecules and a barrage against infection by intracellular pathogens. The PM is also directly connected to intracellular membrane traffic via *endo-* and exocytosis. Not only is the composition of the PM very heterogeneous with several hundred different lipid species, transmembrane proteins and peripheral proteins (Goodwin et al., 2005; Mayor and Maxfield, 1995). There is also the subcortical actin attached to the inner side of the PM via phosphatidylinositol-binding proteins, which allow the cytoskeleton to exert forces to the PM and thereby to control membrane curvature and induce membrane ruffling (Fujimoto et al., 2000; Ganguly et al., 2008; van Rheenen and Jalink, 2002; Kwik et al., 2003a; Boucrot et al., 2006; Mueller et al., 2011). On the other side, we have the extracellular oriented glycocalyx, allowing for anchoring cells in their 3D environment but also for cell signalling and control of membrane bending (Kultti et al., 2006). Together with the dynamically regulated solute and ion composition over the cell border, the PM is of extreme complexity and its analysis asks for a large number of sophisticated experimental approaches. In fact, a prerequisite for new insight into architecture, dynamics and function of the PM is often the establishment of a new experimental technique with a better temporal and/or spatial resolution or with improved sensitivity and specificity in tracking particular PM components. For example, the classical view of the PM was that of a largely homogeneous phospholipid-protein assembly (Edidin, 2003). With the development of high resolution electron microscopy (EM) and the invention of highly sensitive fluorescence labeling

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and imaging over the last 30 years, we now know that the PM is spatially and temporarily heterogeneous. Several models or hypotheses have been put forward to characterize this heterogeneity, and all of them include cholesterol as an important player in PM organization: one model assumes a lipid-based domain architecture in so-called 'rafts' stabilized by specific sphingolipid-cholesterol interactions (Simons and Ikonen, 1997; Mukherjee and Maxfield, 2004). This 'raft' hypothesis is based on physicochemical studies carried out earlier in model membrane mixtures, where it was shown that cholesterol mediates liquid–liquid immiscibility of saturated and unsaturated phospho- and sphingolipids. Evidence for specific interaction of cholesterol with sphingolipids has been provided but is not undisputed, as recently reviewed in detail (Ohvo-Rekila et al., 2002; Ridgway, 2000; Wüstner and Solanko, 2015). The phase rich in cholesterol and saturated lipid species is more ordered (i.e. cholesterol and fatty acyl chains are aligned parallel to the membrane normal) but still fluid with significant lateral diffusion, and it is therefore called liquid ordered (lo) phase. The cholesterol-poor phase harbouring the majority of unsaturated lipid species in the mixture is called the liquid disordered (ld) phase. With the observation that assemblies of PM components which cannot be extracted from cells by certain detergents such as Triton X100 on ice have similar biophysical properties as the lo phase in model membranes, the idea was born that lo-type domains floating as small rafts exist in the native PM of living cells (Ahmed et al., 1997). For a while after this observation, all proteins and lipids being resistant to isolation from the PM by this method (i.e. resistant to cold detergent extraction, so-called detergent resistant membranes, DRMs) had been identified as being 'raft' components in the PM of living cells (Simons and Ikonen, 1997). This included cholesterol and saturated lipids, especially sphingolipids, as well as certain proteins being attached to the membrane via a lipid anchor, as GPI-anchored proteins and prenylated Ras proteins (Simons and Ikonen, 1997; Kenworthy, 2008). Indeed the correlation between resistance to detergent extraction and properties or composition of the lo phase in model membranes was strong, but careful control studies demonstrated that the majority of the PM is resistant to detergent, and that the detergents themselves induce further clustering and thereby enrichment of certain membrane components in the DRMs (Mayor and Maxfield, 1995; Heerklotz, 2002). From that, it was questioned that DRMs resemble any native structure existing in the PM before detergent treatment. It became apparent that the PM as a whole shares many properties with the lo phase which is little surprising given its high content of cholesterol and saturated lipid species (Mukherjee and Maxfield, 2004; Munro, 2003). On the other hand, the protein density in cellular membranes has been estimated to be about 30,000 per μm^2, which provides space only for a few rings of lipids between individual proteins (Jacobson et al., 2007). This is very different from model membrane systems. In later studies other methods have been employed to determine the existence and eventual properties of rafts and other lipid-based membrane domains. Examples are optical trapping of GPI-anchored proteins, analysis of spectral shifts of environmentally sensitive fluorescence probes such as Laurdan and partition preference of probes and proteins into vesicles, directly formed from the PM (so called giant unilamellar PM vesicles, GPMVs) (Pralle et al., 2000; Simons and Gerl, 2010; Bagatolli, 2006). GPMVs are similar to membrane blebs found in apoptotic and cholesterol-overloaded cells as well as in cells with disrupted cytoskeleton (Tank et al., 1982; Wüstner, 2008). However, in contrast to blebs which resemble the intact PM just without cytoskeleton support (Tank et al., 1982), formation of GPMVs requires adding membrane intercalators such as formaldehyde and dithiotreitol, and phase segregation of 'raft-markers' in those GPMVs is mostly found after chemical cross-linking or after prolonged incubation at 2 °C. Laurdan is a versatile probe since it partitions roughly equally between ld and lo phase in model membranes, and its emission properties directly report about water accessibility of the bilayer, often measured as generalized polarization (GP) value (Bagatolli, 2006, 2012). However, while the underlying phenomena for Laurdan's emission properties are well understood in model membranes, the situation is more complex and much less defined in cellular membranes. For example, the GP value of Laurdan in the PM can be affected by bilayer tension, curvature and protein content, making its interpretation less straightforward (Bagatolli, 2006; Zhang et al., 2006). So, the question is to what extent the results obtained with model membranes or with isolated cell membranes after heavy experimental manipulation, such as in GPMVs, can be translated into knowledge about the dynamics or distribution of the investigated protein or lipid components in the intact PM of living cells. Another membrane model, called the "membrane skeleton fence model", is based on extensive single particle tracking (SPT) and fluorescence recovery after photobleaching (FRAP) studies in living cells and assumes, that the membrane-attached cytoskeleton plays a crucial role in compartmentalization of the PM (Subczynski and Kusumi, 2003; Sheetz, 2001; Koppel et al., 1981). Thus, also in this model the PM is compartmentalized but the driving forces are the interactions of transmembrane and peripheral membrane proteins with the cortical cytoskeleton, mainly with actin (Sheetz, 2001; Ritchie et al., 2003a). There exist various modifications and refinements of both models, which cannot all be discussed here. The major conceptual difference between the raft hypothesis and the "membrane skeleton fence model" is that in the former preferred interactions with sphingolipids and cholesterol act as organization principle, while in the latter protein–protein interactions provide the driving force for the heterogeneous organization of the PM. We will see in the following sections how experimental studies help in discriminating between both hypotheses.

1.2. The plasma membrane is not flat: effects of surface topography

Both models have in common that the PM is assumed to be a flat 2-dimensional (2D) structure with zero curvature, and that heterogeneity is solely caused by differential interactions of the membrane constituents. Recent studies using atomic force microscopy (AFM), scanning electron microscopy (SEM) or scanning surface confocal microscopy have clearly established that the cell surface has a rough topography with filopodia and microvilli bridging a spatial scale of 100 – 500 nm up to several micrometers (Braet et al., 1998; Gorelik et al., 2003). Moreover, theoretical studies have shown that lateral diffusion of particles on a 2D surface is very sensitive to irregularities and fluctuations of the surface geometry and that this influence grows exponentially with time (Gov, 2006; Chevalier and Debbasch, 2008). For example, the projected self-diffusion of a probe in the xy-plane can differ by several orders of magnitude from the curvilinear self-diffusion on the real surface topography depending on the flexibility of the bilayer and thereby the amplitude of surface protrusions (Chevalier and Debbasch, 2008; Naji and Brown, 2007). Accordingly, transient trapping of PM components, for example membrane proteins but also tagged lipid probes, in SPT experiments can be a consequence of local surface protrusions, which take the particle longer to explore by purely Brownian motion than a flat surface (Hall, 2008; Adler et al., 2010).

1.3. The role of cholesterol: methods of its manipulation in the plasma membrane

A straightforward way of assessing the role played by cholesterol in regulating PM organizations is to change its