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Review

Ionic control of the metastable inner leaflet of the plasma membrane: Fusions natural and artefactual

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ABSTRACT

The phospholipids of the inner and outer leaflets of the plasma membrane face chemically very different environments, and are specialized to serve different needs. While lipids of the outer leaflet are inherently stable in a lamellar (bilayer) phase, the main lipid of the inner layer, phosphatidylethanolamine (PE), does not form a lamellar phase unless evenly mixed with phosphatidylserine (PS^-). This mixture can be readily perturbed by factors that include an influx of Ca^{2+} that chelates the negatively charged PS^-, thereby destabilizing PE. The implications of this metastability of the inner leaflet for vesicular trafficking, and experimentally for the isolation of detergent-resistant membrane domains (DRMs) at physiological temperature, are considered.

1. Asymmetric challenges of the plasma membrane

The thin lipid bilayer of surface membrane interfaces with two very different environments in mammalian cells. The outer leaflet faces an oxidizing environment, with Ca^{2+} the major divalent cation regulated around 1.2 mM, and Na^+ the major monovalent cation. The inner leaflet faces a reductive environment with Mg^{2+} and K^+ the major cations, and basal Ca^{2+} held at sub-μM levels. Proteins destined to be exposed to the external environment are synthesized in the lumen of membrane-bound compartments (endoplasmic reticulum, Golgi) in which a Ca^{2+} rich, oxidizing environment is maintained, allowing disulfide bonds to form and carbohydrate chains added to provide a measure of redox protection. Material moves between these compartments within vesicles that preserve the oxidizing environment in their lumens, so the chemical asymmetry of the cell surface is maintained between this biosynthetic chain of organelles deep within the cell. It is also largely maintained in organelles that sample and degrade material ingested from the external environment (endosomes, lysosomes), again connected by vesicle trafficking to maintain the surface asymmetry.

This standard cell biology must be familiar to all. What is not so familiar is how the different outer and inner leaflet lipids are specialized to face these different environments. This review will highlight some structural properties of these lipids that serve the different functions of the inner and outer leaflet, and in particular support the intensive vesicle trafficking mediated by the inner leaflet. It will also consider how these properties influence the isolation of membrane fragments, specifically in the case of detergent-resistant membranes (DRMs) which, in the absence of any alternative, are taken to biochemically represent the composition of lipid 'rafts' on cells.

2. Structural and functional stability of the outer leaflet

The two lipids that dominate the extracellular leaflet of the plasma membrane, phosphatidylcholine (PC) and sphingomyelin (SM), have the same headgroup, phosphocholine, that resists oxidation [1,2] and is not cross-linked by Ca^{2+} [3,4]. Being zwitterionic, phosphocholine headgroups lie down on the surface of the membrane in reciprocal orientations to appose their opposite charges and thereby cover an area of bilayer to match that of the underlying volume occupied by their mobile fatty acid chains [5,6]. This size matching of surface headgroup to the underlying volume gives these lipids a cylinder shape that forms a stable lamellar phase in water [7,8]. SM differs from PC in having its fatty acid chain bonded by an amide linkage, an electron donor and acceptor that allows direct hydrogen bonding between SM molecules to form labile clusters of several SM molecules in the

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membrane [9,10], adding an extra stability not provided by the solely electron donating ester linkage of glycerolipids.

The inherently stable monolayer produced by phosphocholine-headed lipids is further augmented by the condensation of cholesterol with saturated PC and SM to produce separate nm-scale domains ('rafts') in a liquid-ordered phase (Lo) floating in the liquid disordered (Ld) phase of unsaturated lipids [11,12]. The underlying drive may be the need to shield hydrophobic cholesterol from water under the 'umbrella' of the phosphocholine headgroup [13], although the close interaction of the rigid sterol rings with saturated hydrocarbon chains is exothermic [14]. This tight packing of sterol to lipid makes the membrane more ordered, hydrophobic [15,16] and less elastic [17]. Glycosphingolipids, the third class of outer leaflet lipid, add redox-buffering carbohydrate groups and form nanodomains that provide another level of lateral heterogeneity for lipids and proteins on the cell surface [18]. Their large headgroups add strong positive curvature to the membrane [19].

Thus outer leaflet lipids form a stable protective layer against the harsh external environment. Individual lipids are in a constant state of flux as they are modified for signaling (e.g. Sonnino et al. and van Meer and Hoetzl), pass between Lo domains on a μs time scale [20] and are disrupted continually by endocytosis and the arrival of new biosynthetic vesicles. But they play a secondary role in this vesicular trafficking, which is orchestrated on the cytoplasmic side. The outer leaflet is made for stability, the cell's first line of defence against a chemically, and sometimes biologically, hostile environment.

3. Metastability of the inner leaflet

Before touching upon the biophysics of inner leaflet lipids, it would help to observe Supplementary Film 1 that uses the Richardson RTM microscope to show, purely by high resolution phase contrast optics, the extent of vesicular trafficking along a bifurcated axon of an adult sensory neuron actively growing in tissue culture. Mitochondria and lysosomes are readily seen, but it is the haze of barely distinguishable (60–100 nm) transport vesicles moving in each direction along the axon that these remarkable optics reveal. It is this intense vesicular transport that the metastability of the inner leaflet serves.

PE, the major inner leaflet lipid, does not itself form a lamellar (membrane) phase since its headgroup is too small to cover its fatty acid chains, giving it a cone shape [7,8]. It will normally form an inverse hexagonal phase (inverted micelles) in water. To form a lamellar phase, it mixes with 10% PS^-, whose larger headgroup has a complementary shape (inverted cone) so that the mixture fills out a balanced lipid monolayer [21–23]. Electrostatic repulsion ensures that negatively charged PS^- is uniformly spread over the inner leaflet.

Many factors trigger the transition of PE between lamellar and hexagonal phases. The one that concerns us here is Ca^{2+}, the extracellular cation kept at a basal <μM concentration intracellularly, from which it rises sharply in response to signaling in spatially and temporally-restricted peaks or oscillations [24]. Ca^{2+} (but not the intracellular cation Mg^{2+}) cross-links PS^- with low μM affinity [25], clustering this lipid in the plane of the membrane away from PE. The instability of naked PE in the membrane leaflet can be resolved by fusing with another membrane [3,25–27]. Cholesterol condensed with saturated PE/PS^- [28] further sensitizes PE to more readily revert to an hexagonal phase [29]. Some Ca^{2+}-activated proteins involved in vesicular trafficking, such as the annexins that also cross-link multiple PS^- molecules [30], presumably augment this mechanism of destabilizing PE.

It was quickly realized that Ca^{2+}-PS^- mediated destabilization of PE would contribute to vesicle fusion [31]. In a more detailed analysis [32], Zimmerberg and Chernomordik note that lipid constraints determine the energetics of vesicular fusion; protein-induced stress on the membrane directs the process [33]. Both PE and PS^- are markedly less hydrated than PC [34] to make the cytoplasmic face dryer and more fusogenic than the external surface of the cell. Energetically, the essential fusion intermediates are membrane stalks formed between apposed membranes, in which non-bilayer lipids such as PE play a vital role in providing the required negative membrane curvature [33,35].

4. Isolation of detergent-resistant membranes (DRMs)

Could ionic destabilization of the inner leaflet sabotage attempts to isolate lipid 'rafts' as DRMs? This method seeks to isolate raft membrane from cells by exploiting the ability of compact Lo domains to exclude non-ionic detergents, that solubilize instead the surrounding Ld membrane to release DRMs to float at low density upon gradient density ultracentrifugation [36]. The contention that these DRMs sample raft membrane has been criticized at many levels [37], the most telling of which is arguably why DRMs can only be isolated at 4 °C when these compact, Lo 'raft' domains are present at physiological temperature?

A feature of the lipid composition of DRMs isolated at 4 °C with the benchmark detergent Triton X-100 is that they have a marked imbalance of outer, over inner, leaflet lipids [38–40], implying that this method strips off inner leaflet lipids. The resultant monolayer fragments of outer leaflet must be very unstable in water, and presumably fuse to give large bilayer mosaics of patches of outer leaflet. Experimentally, Triton X-100 DRMs merge proteins from totally different membranes [41] and have outer leaflet proteins facing externally and internally [42]. Stripping off inner leaflet lipids appears to be particularly a problem with Triton X-100 that has a short, bulky hydrophobic tail. Brij 96, with a longer (C18) alkyl chain, isolates balanced proportions of inner and outer leaflet lipids [40,43] with increased recovery of lipid-anchored raft proteins of the inner leaflet [44]; further, it does not fuse different membranes or scramble membrane asymmetry [41,42].

Could a major problem be that addition of detergent releases Ca^{2+} stores from endoplasmic reticulum, mitochondria and lysosomes, and this destabilizes the inner leaflet lipids making them selectively soluble? This would appear to be a significant factor, because solubilization of membrane in either Brij 96 or Triton X-100 using a buffer that chelates Ca^{2+} and contains Mg^{2+} and K^+ allows DRMs to be isolated at 37 °C (indeed, at temperatures up to 55 °C [45] as expected given the thermal stability of Lo phases [46,47]). DRMs isolated in Brij 96 at 37 °C are slightly smaller (diameter of 120–180 nm by dynamic light scattering) and thicker (46.4 nm by small angle neutron scattering) than those isolated at 4 °C [48]. Glycosylphosphatidylinositol (GPI)-anchored proteins Thy-1 and cellular prion protein (PrP^C), found on the neuronal surface in distinct although often adjacent clusters and with very different trafficking properties [41,49,50], can be isolated separately by either immunoaffinity purification or by their different density on the sucrose gradient; and each is associated with different proteins attached to their cytoplasmic surface, reflecting their different biology [45]. Asymmetry of the membrane is preserved in the DRM vesicles [45]. This combination of properties suggests that the membrane domains sampled by DRMs at 37 °C are discrete, 20–200 nm-scale regions of the cell surface.

5. So do these 37 °C DRMs isolate 'raft' membrane?

This is all very promising, and suggests the application of simple biophysical principles to the isolation of membrane fragments may enable the isolation of the elusive 'raft'. However, the results chal-