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Current Opinion in Colloid and Interface Science 8 (2004) 459–468

Current Opinion in COLLOID and INTERFACE SCIENCE

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Cholesterol–phospholipid interactions, the liquid-ordered phase and lipid rafts in model and biological membranes

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Abstract

The existence of relatively large and long-lived detergent-insoluble, sphingolipid- and cholesterol-enriched, liquid-ordered lipid raft domains in the plasma membranes of eukaryotic cells has become widely accepted. However, we believe that the evidence for their existence is not compelling despite extensive work on both lipid bilayer model and biological membranes. We review here the results of recent studies, which in our view call into question the existence of lipid rafts in membranes, at least in the form commonly depicted.

Keywords: Cholesterol–phospholipid interactions; Liquid-ordered phase and lipid rafts; Model and biological membranes

1. Introduction

The membranes of eukaryotic cells contain three classes of lipids: glycerophospholipids, sphingolipids, and cholesterol or a closely-related sterol (see Fig. 1). The glycerophospholipids consist of a glycerol backbone, two ester-linked fatty acyl chains, and a phosphorylated alcohol, typically phosphorylcholine, phosphorylethanolamine or phosphorylserine. The fatty acyl chains usually contain an even number of carbon atoms, typically between 16 and 20 in total, with one hydrocarbon chain being saturated and the other unsaturated. The sphingolipids are based on the more complex alcohol sphingosine and contain a single amide-linked fatty acyl chain, which is usually saturated and may contain up to 24 carbon atoms, and either a phosphorylated alcohol (usually phosphorylcholine), or one or more sugar molecules linked to the hydroxyl terminus of the sphingosine backbone. The second hydrocarbon chain of sphingolipids is actually the non-polar portion of the sphingosine molecule itself, and unlike the glycerophospholipids, an unesterified hydroxy group is present at C3, which can act as a hydrogen bond donor and acceptor. Finally, cholesterol consists of a fused cyclic four-ring structure containing a single polar hydroxyl group and an isooctyl side chain, the cyclic ring system being essentially planar and rigid. The lipid compositions of the plasma membranes of eukaryotic cells are unusual in containing higher levels of sphingolipids and cholesterol, and lower levels of glycerophospholipids than do other cell membrane systems. Although the relative proportions of these three lipid classes vary according to species and cell type in vertebrates, cholesterol is typically present at levels of 30–40 mol.%, sphingolipids at levels of 10–20 mol.%, and glycerophospholipids at levels of 40–60 mol.% of the total plasma membrane lipids (see Ref. [1]).

Another important property of the plasma membranes of human and animal cells is that they exhibit transverse lipid compositional asymmetry. That is, the lipid composition of the outer and inner monolayers or leaflets of the lipid bilayer are different. Specifically, the zwitterionic glycerophospholipid phosphatidylcholine (PC) and the zwitterionic phosphosphingolipid sphingomyelin (SM) are enriched in the outer monolayer, while the zwitterionic glycerophospholipid phosphatidylethanolamine (PE) and the anionic glycerophospholipid phosphatidylserine (PS) are enriched in or restricted to, the

Abbreviations: PC, phosphatidylcholine; SM, sphingomyelin; GSL, glycosphingolipid; PE, phosphatidylethanolamine; PS, phosphatidylserine; DOPC, dioleoylphosphatidylcholine; POPC, 1-palmitoyl, 2-oleoylphosphatidylcholine; SOPC, 1-stearoyl, 2-oleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; L_α, lamellar liquid-crystalline; L_β, lamellar gel; L_d, lamellar liquid-disordered; L_o, lamellar liquid-ordered; DRM, detergent-resistant membranes; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance.

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doi:10.1016/j.cocis.2004.01.007

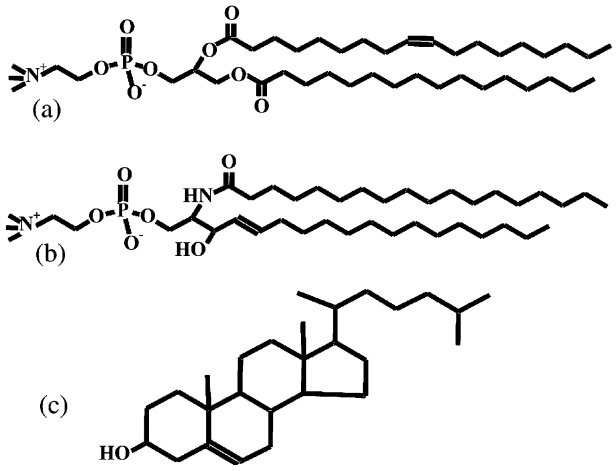

Fig. 1. The chemical structures of representatives of the three major classes of lipids found in eukaryotic cells: phosphatidylcholine, a glycerophospholipid (a); sphingomyelin, a sphingophospholipid (b); and cholesterol, the major sterol of higher animals (c).

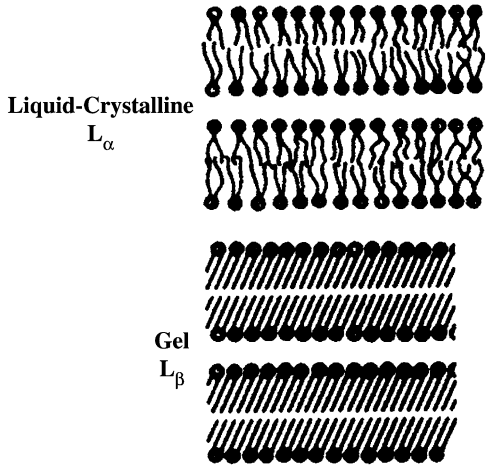

Fig. 2. Schematic diagrams of the structures of lipid bilayers in the liquid-crystalline or L_α phase (top) and in the gel or L_β phase (bottom). The structure of the liquid-ordered or L_o phase is perceived as an intermediate structure.

inner monolayer, respectively (see Ref. [2]). The transverse distribution of cholesterol is less well understood, but because of its high rate of flip-flop between the inner and outer monolayers and because roughly equal amounts of glycerol- and sphingolipids are present in each leaflet, cholesterol is usually assumed to be roughly equally distributed across the lipid bilayer [2]. However, in view of the relatively higher affinity of cholesterol for SM and PC as compared to PS and PE (see below), it is possible that cholesterol could be enriched to some degree in the outer monolayer.

Since cholesterol is present in high levels in animal plasma membranes, it is important to consider its effects on the structure and organization of bilayers composed of glycerol- and sphingophospholipids. In the absence of cholesterol, lipid bilayers can exist in one of two physical states, the gel (L_β) state at lower temperatures and the liquid-crystalline (L_α) state at higher temperatures (see Fig. 2). In the L_β state, the phospholipid hydrocarbon chains are in the fully extended all-trans conformation, the cross-sectional area of the phospholipid molecules is minimal, the thickness of the phospholipid bilayer is maximal, and both intra- and intermolecular motion are severely restricted. In the L_α state, the phospholipid hydrocarbon chains contain a number of gauche rotational conformers, the cross-sectional area of the lipid molecules increase considerably, the phospholipid bilayer thins substantially, and relatively high rates of both intra- and intermolecular motion are present (see Ref. [3]). Numerous biophysical studies of the membranes of living cells not containing cholesterol have shown that under physiologically relevant conditions, the lipid bilayer exists at least predominantly if not exclusively in the L_α phase, and that this phase is required for normal cell growth and membrane function to occur (see Ref. [4]). The mixture of glycerol- and sphingolipids of animal plasma membranes would be expected to exist as a mixture of L_α and L_β phases in the absence of cholesterol, due to the lower L_β/L_α phase transition temperatures (approx. 0 °C) of the unsaturated glycerophospholipids and the higher phase transition temperatures of the more saturated sphingolipids (approx. 37 °C) [5]. In fact, it has been proposed that one of the roles of cholesterol in animal plasma membranes is to reduce the intrinsic tendency of glycerophospholipids and sphingolipids to form separate L_α and L_β phases [5], in part by inducing the formation of an intermediate phase state, as described below. Indeed, a recent study has found that severe cholesterol depletion induces large scale domain separation, presumably consisting of glycerophospholipid-enriched L_α and sphingolipid-enriched L_β phases, in the plasma membranes of living cells [6]. In this regard, it is interesting to note that in the lipid raft hypothesis, cholesterol is also proposed to facilitate a fluid–fluid lateral phase separation of glycerophospholipid- and sphingolipid-enriched phases into separate domains, while simultaneously inhibiting the formation of sphingolipid-enriched L_β phases.

2. The liquid-ordered phase

A huge literature exists on the effects of adding cholesterol to glycerophospholipid and sphingolipid bilayers (see Refs. [7–11]). The major effects of the incorporation of progressively increasing amounts of cholesterol into lipid bilayers are to: broaden and reduce the enthalpy of the L_β/L_α phase transition, eliminating it entirely at 50 mol.% cholesterol; to decrease (increase) the cross-sectional area of the phospholipid molecule in

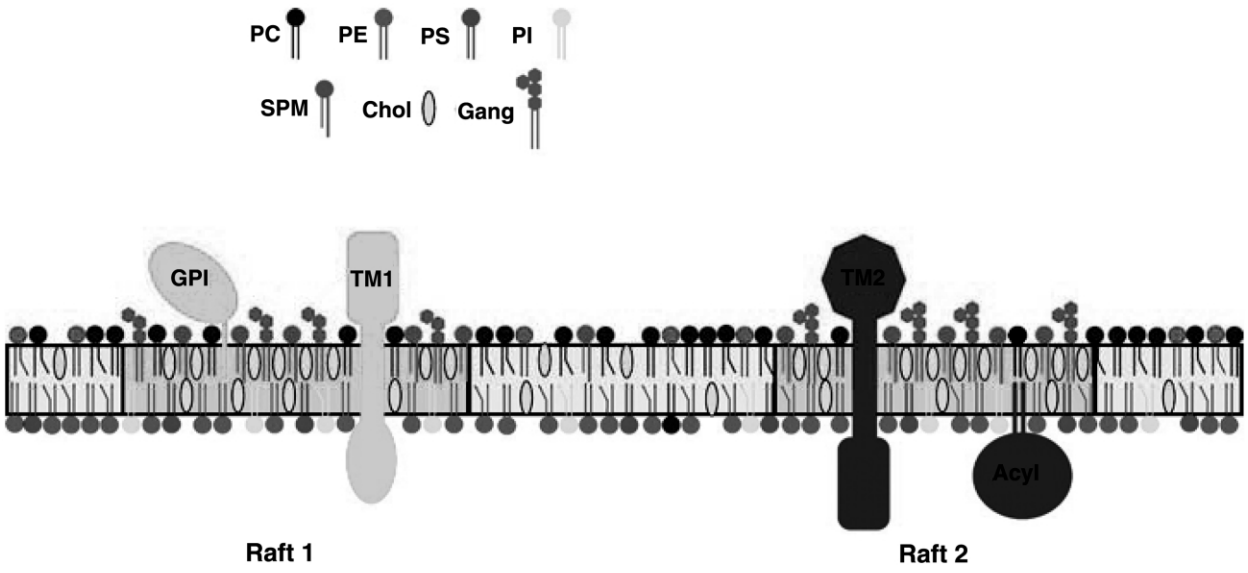

Fig. 3. Schematic diagram illustrating the proposed organization of lipid rafts in cell membranes. Lipid rafts (blue bilayer) are perceived as specialized membrane domains containing high concentrations of cholesterol, sphingomyelin, and gangliosides. They are also enriched in phospholipids that contain saturated fatty acyl chains (straight lines in lipid tails). This composition results in lateral phase separation and the generation of a liquid-ordered domain. Bulk plasma membrane (gray) contains less cholesterol, sphingomyelin, and gangliosides, and more phospholipids with unsaturated acyl chains. As a result, it is more fluid than lipid rafts. A variety of proteins partition into lipid rafts: glycosylphosphatidylinositol-anchored proteins; transmembrane proteins (TM); and dually acylated proteins (Acyl). As shown in the diagram, not all lipid rafts have the identical protein or lipid composition (Raft 1 vs. Raft 2). Reproduced from [17] with permission.

the $L_\alpha(L_\beta)$ state; to increase (decrease) the thickness of the phospholipid bilayer in the $L_\alpha(L_\beta)$ state; and to increase (decrease) the orientational order and decrease (increase) the rates of motion of the hydrocarbon chains in the $L_\alpha(L_\beta)$ state. The progressive addition of cholesterol also increases the mechanical rigidity and cohesiveness and reduces the permeability of phospholipid bilayers in the L_α state, while also reducing the rotational and lateral diffusion rates of the phospholipid molecules somewhat. In summary, then, the addition of increasing amounts of cholesterol progressively converts the L_α and L_β states of phospholipid bilayers to a state which has properties generally intermediate between those of solid (L_β) and fluid (L_α) phospholipid bilayers. Hence the term 'liquid-ordered' (or perhaps better 'liquid-crystalline ordered') phase, denoted as L_o, to describe this intermediate state has been appropriated [12], while the L_α state is termed the 'liquid-disordered' (better the 'liquid-crystalline disordered') phase, denoted as L_d.

3. The lipid raft hypothesis

An exciting new development in the field of membrane biology in recent years has been the hypothesis that the plasma and possibly other membranes of animal cells may contain laterally segregated lipid domains known as 'lipid rafts' (see Refs. [13,14]). Lipid rafts were originally conceived as relatively long-lived domains of appreciable size, which are depleted in phospholipids and many transmembrane proteins but which are enriched in cholesterol, sphingolipids and lipid-anchored proteins (see Fig. 3). These discrete lipid raft domains were postulated to exist in a relatively ordered lamellar liquid-crystalline phase (the L_o phase) while the continuous non-raft regions of the membrane exist in a relatively disordered lamellar liquid-crystalline phase (the L_α or L_d phase). Moreover, the different physical properties of the L_o and L_d phases were postulated to play a key role in the lateral segregation of the various classes of membrane proteins. Lipid rafts were also proposed to play important roles in the sorting and targeting of lipids and proteins to various membrane systems, in signal transduction across the plasma membrane, and in many other cellular functions.

Three of the key pieces of in vivo evidence for the existence of lipid rafts in biological membranes have been the isolation of so-called detergent-resistant membrane (DRM) fractions from human and animal cells, the disruption of the function of human and animal plasma membranes by cholesterol depletion, and the non-random lateral distributions raft-associated proteins in such membranes. As well, the biophysical demonstration of the coexistence of L_d and L_o phases in lipid monolayer or bilayer model membranes with lipid compositions thought to reflect those of the lipid raft domains of plasma membranes has lent support to this hypothesis. However, in our view, the isolation of DRMs from whole human or animal cells, the inhibition of

membrane function by cholesterol depletion, and the non-random distribution of membrane proteins, provide at best, weak support for the existence of lipid rafts in their plasma membranes. Similarly, we feel that the case for the presence of coexisting long-lived L_d and L_o domains of appreciable size in actual animal or human plasma membranes is also unconvincing, although such domains clearly exist in the canonical ternary lipid mixture thought to reflect the composition of lipid rafts. In this brief critical review, we consider primarily the evidence for the existence of lipid rafts arising from biophysical studies of lipid model membranes. However, Edidin [15•] and Munro [16•] have recently published excellent more comprehensive review articles considering the structural and functional evidence for lipid rafts in both model and biological membranes, and Pike [17] has recently published a valuable review focusing primarily on the isolation and physical characteristics of lipid rafts and their roles in cellular signaling. We will also briefly consider the in vivo evidence for the existence of lipid rafts in the plasma membranes of human and animal cells near the end of this present article.

4. The detergent solubility of membrane lipids

The hypothesis that cholesterol- and sphingomyelin-enriched lipid rafts exist in biological membranes was initially based primarily on the observation of so-called DRMs [13,14]. DRMs were defined operationally as membrane fractions which resist solubilization by the non-ionic detergent Triton X-100 (TX) at 4 °C. However, there are a host of potential problems with this approach, not the least of which is the requirement for detergent treatment at very low temperatures, which will certainly significantly alter the physical state of the lipids of plasma membranes and which can accentuate or even induce lateral lipid phase separations, which may not have been present at 37 °C. Moreover, the lipid and protein compositions, and even whether or not DRMs are observed, depend strongly on the nature of the non-ionic detergent used and on its concentration, on the time and temperature of detergent extraction, and other variables (see Refs. [17–19]). In addition, the DRMs isolated from sucrose gradients are a heterogeneous collection of vesicles of widely varying size, or sometimes even sheets of membrane [20]. Moreover, although a fair amount is known about how TX and other detergents solubilize simple lipid model membranes (see below), the actual molecular mechanisms by which non-ionic detergents solubilize morphologically and biochemically complex whole cells containing many different membrane systems are largely unknown. These complicating factors strongly suggest that extreme caution should be used in equating the isolation of DRMs with the existence of lipid rafts in vivo.

A major step forward in understanding the process of lipid model membrane solubilization by TX has recently been provided by Heerklotz [21••], who studied temperature-dependent phase transitions in vesicles containing the canonical lipid raft equimolar mixture of POPC, SM and cholesterol (see below) by DSC and pressure perturbation calorimetry. In addition, transitions between various lamellar and micellar phases induced by TX addition were studied by isothermal titration calorimetry and ^{31}P-NMR spectroscopy. An important finding of this study is that TX disorders the L_d phase and orders the L_o phase in model membranes containing both phases, by partitioning selectively into the more disordered membrane phase, and that this effect is accentuated by cooling. Thus, the addition of TX actually promotes L_o domain formation in lipid vesicles initially containing a mixture of L_d and L_o phases. Another important observation is that the composition of the DRM fraction is dependent on both the temperature of treatment and the detergent concentration. As well, the L_o domain can be induced by TX addition and cooling even in a homogeneous lipid mixture initially existing only in the L_d phase. The major conclusion of this study is that DRMs, even in relatively compositionally simple lipid model membranes, should not be expected to resemble lipid raft domains in abundance, size or composition, or even to imply the existence of lipid raft domains prior to detergent treatment.

Heerklotz et al. have recently extended their earlier work on POPC/SM/cholesterol equimolar mixtures to examine the sensitivity of coexisting L_d and L_o domains to perturbations resulting from the addition of TX [22••]. Their major new finding is that the small transition enthalpy between the L_d and L_o domains in such ternary lipid mixtures makes domain formation or disintegration very sensitive to small perturbations included by temperature or pressure changes, or by the addition of detergents or possibly even fluorescent probes. Since SM- and cholesterol-rich L_o domains in such model raft membranes are only marginally stable under physiologically relevant conditions, extreme care is required in using a number of techniques commonly used to study lipid rafts in model or biological membranes, most especially the use of detergents and low temperature in isolating DRMs.

Although it would certainly be desirable to more fully understand the process of the detergent solubilization of the lipids and proteins of whole animal cells by TX and other non-ionic detergents, such a complex and daunting task is unlikely to be completed in the near future. Moreover, even if this task were successfully accomplished, it is unlikely to completely resolve the issue of whether the formation of DRMs has direct relevance to the lipid organization of intact animal plasma membranes. Perhaps a step forward in this direction, however, would be to apply TX solubilization procedures to

isolated plasma membranes, possibly to the well-studied human erythrocyte membrane, to determine whether or not a DRM membrane fraction could be isolated, and if so, to determine how its lipid composition would relate to that of the whole membrane. Still, the use of detergents to study the organization of biological membranes is inherently fraught with great difficulties and will likely never be a definitive technique for membrane research.

5. The lipid composition of rafts

The canonical 'lipid raft' mixture utilized in many studies of lipid model membranes is made up of an equimolar mixture of PC (usually DOPC), SM and cholesterol. This composition is based primarily on a lipid compositional analysis of whole Madin–Darby canine kidney (MDCK) cells and TX-insoluble DRMs, the latter presumably representing the L_o domains present in the putative lipid rafts of the plasma membrane [20], although as discussed above, the lipid composition of DRMs depends on the detergent used and a host of other variables. In fact, these particular MDCK DRMs contain 34.9 mol.% glycerophospholipid, 32.5 mol.% sphingolipid and 32.7 mol.% cholesterol. Thus, as these authors correctly point out, DRMs are indeed depleted in glycerophospholipids and enriched in sphingolipids and cholesterol compared to the lipids of whole cells. However, since whole cells contain other membrane systems beside the plasma membrane, many of which contain little if any sphingolipid or cholesterol, and MDCK cells also contain almost 25 mol.% triglyceride, a lipid not even found in appreciable amounts in cell membranes, the biological significance of this comparison between the lipid compositions of whole cells and DRMs is questionable. A more relevant comparison would be between the lipid composition of the DRMs and the plasma membranes of these cells, where the lipid rafts are postulated to exist. Unfortunately, the lipid composition of purified plasma membranes from MDCK cells seems not to have been determined. However, if one compares the lipid compositions of the DRMs of MDCK cells with that of typical mammalian plasma membranes (see Ref. [1]), then the former is indeed moderately depleted in glycerophospholipids and markedly enriched in GSLs but contains rather typical amounts of SM and cholesterol. Thus, these DRMs ('lipid rafts') are not SM- and cholesterol-enriched compared to plasma membranes generally.

There are other disturbing aspects of the canonical lipid raft mixture. The DRMs from MDCK cells actually contain four glycerophospholipids, with PE (17.6 mol.%) and PC (12.4 mol.%) being the two major components [20]. Leaving aside for the moment the troubling finding that the levels of PE were reported to be considerably higher than those of PC in these whole MDCK cells (the typical finding is the reverse), the choice of PC as the representative glycerophospholipid of rafts is arbitrary, since PE is actually the major glycerophospholipid present in these particular DRMs. The reason that PC rather than PE was selected as the canonical lift raft glycerophospholipid representative seems to be that PC is enriched and PE is depleted in the outer monolayer of mammalian plasma membranes, and since lipid rafts were first postulated to exist only in the outer monolayer, PC rather than PE was selected. However, since more recent work suggests that in both lipid model and biological membranes, lipid rafts are present in both monolayers of the lipid bilayer and are coupled across the bilayer (see Refs. [15•,16•,17]), this rationale for the choice of PC over PE is questionable. Similarly, in the DRMs, total GSLs make up 21.4 mol.% and SM only 11.1 mol.% of the total lipids, yet SM was selected as the representative sphingolipid, despite the fact that GSLs are more highly enriched in the outer monolayer of mammalian plasma membranes than are SMs (see Ref. [2]).

Even assuming for the moment that the choice of PC as a single glycerophospholipid representative is nevertheless reasonable, the selection of DOPC as a representative molecular species for the PCs of mammalian plasma membranes certainly is not. In fact, glycerophospholipids which contain either two unsaturated or two saturated fatty acid chains are scarce in membranes and molecular species of glycerophospholipids with a saturated chain on the first position of the glycerol backbone and an unsaturated chain on the second position overwhelmingly predominate (see Ref. [23]). Thus, the selection of one of the more common molecular species of PC, such as POPC or SOPC, would be considerably more appropriate and biologically relevant. Moreover, DOPC has a much lower intrinsic L_β/L_α phase transition temperature (approx. -18 °C, see Ref. [24]) than do POPC and SOPC (approx. -3 °C and $+9$ °C, respectively, see Refs. [5,25]). Since SMs have L_β/L_α phase transition temperatures of approximately 37 °C, and since the miscibility of two lipids species in either the L_β or L_α phases decreases markedly as the difference in their L_β/L_α phase transition temperatures increase [5,25], the utilization of DOPC rather than POPC or SOPC in equimolar mixtures with SM and cholesterol is likely to at least accentuate, if not actually induce, coexisting lipid domains in these model membrane systems which may not in fact exist in animal plasma membranes, particularly at biologically relevant temperatures (37 °C). Moreover, the affinity of DOPC for cholesterol is also considerably lower than that of POPC and SOPC (see below), which will further contribute to the tendency of DOPC and SM for lateral phase separation. In fact, it is much more difficult to observe raft-like domains in POPC-containing mixtures with SM and cholesterol than in DOPC-containing

mixtures, particularly at temperatures near 37 °C (see Ref. [26•]), and would likely be even more difficult in SOPC-containing ternary mixtures. The choice of the diunsaturated DOPC as a model raft glycerophospholipid is also questionable in that in DRMs, the glycerophospholipids present are generally enriched in saturated fatty acids relative to those from whole cells [19,27,28].

One very important final point about the compositional and physical equivalence of DRMs and lipid rafts should be made. If the lipid composition of DRMs does accurately reflect that of the lipid raft domains of plasma membranes and the putative lipid rafts of such membranes do exist exclusively in the L_o phase, then the canonical lipid raft mixture should also exist exclusively in this phase as well. However, the canonical equimolar DOPC/SM/cholesterol model membrane actually exists as a mixture of L_d and L_o phases even at room temperature, and the L_d phase may even predominate at 37 °C (see below). This result indicates either that DRMs have rather different lipid compositions from the lipid rafts of plasma membranes, in which case their relevance to the lipid raft hypothesis is questionable, or that the lipid rafts in such membranes, if they actually exist, are not exclusively in the L_o state. In this regard, studies of lipid bilayer model membranes composed of the actual lipids from animal plasma membranes should be a priority, as well as studies of model membranes accurately reflecting the actual lipid compositions of the inner and outer lipid leaflets of such membranes.

6. Cholesterol–phospholipid phase diagrams

Several investigators have attempted to construct temperature/composition phase diagrams for cholesterol/phospholipid binary mixtures in excess water [12,29,30]. However, by far the most cited phase diagram is that of Vist and Davis [12], who employed DSC and ^{2}H-NMR spectroscopy to determine the phases and phase boundaries of mixtures of cholesterol and chain-perdeuterated DPPC. These investigators identified three different phases which exist below cholesterol concentrations of 20–25 mol.%, depending on temperature: the L_β and L_α phases of DPPC which exist at lower and higher temperatures, respectively, at low cholesterol concentrations, and the L_o phase which coexists with either the L_β or L_α phases at higher cholesterol concentrations. However, at cholesterol concentrations above 20–25 mol.%, only the L_o phase exists at all temperatures. Furthermore, the general form of this phase diagram was proposed to be universal and essentially independent of the fatty acid structure and chain length of the PC molecule [31]. However, we subsequently reported that the eutectic point postulated by Vist and Davis to exist at 7.5 mol.% cholesterol was an artifact arising from a crossover of the sharp and broad components of the DSC thermograms, which arise from DPPC-enriched and cholesterol-enriched domains, respectively, on the temperature scale, thus producing an apparent sharpening of the gel to liquid-crystalline phase transition [30,32]. Thus we proposed that the L_o phase coexists with either the L_β or L_α phase even at very low cholesterol concentrations. Moreover, we also demonstrated that the positions of the phase boundaries in the phase diagram do vary considerably with the fatty acid structure and chain length of the PC. Nevertheless, for the purposes of this review, the important point is that both groups agree that at temperatures above the PC phase transition temperature, varying proportions of L_α (or L_d) and L_o phases exist at all cholesterol concentrations below 20–25 mol.%, but that only the L_o phase exists at higher cholesterol concentrations. Since the general effects of cholesterol on the thermotropic phase behavior and organization of other glycerol and sphingophospholipids appear to be similar to those for DPPC [33–35], one must question, then, whether or not the L_d phase can even exist in plasma membranes containing 30–50 mol.% cholesterol, let alone forming the continuous phase within which the L_o phase of lipid rafts is proposed to exist.

In considering these phospholipid/cholesterol phase diagrams, the question of whether or not the 'intermediate state' of the lipid bilayer actually corresponds to a single thermodynamic phase (the L_o phase) should be considered, because the thermodynamic, structural and dynamic properties of this state often vary continuously, and even smoothly, with variations in the cholesterol content of the lipid bilayer over a wide range of cholesterol concentrations (see Refs. [7–11]]. Although there is good evidence for the existence of two discrete thermodynamic states at lower cholesterol concentrations (see above), the question of whether or not the properties of the compositionally invariant 'intermediate state', which coexists with L_d state at lower cholesterol levels, are identical to those of the compositionally variable 'intermediate state' which exists exclusively at higher cholesterol concentrations, needs to be critically examined. Certainly, the thermodynamic properties associated with the chain-melting phase transition, the area per phospholipid molecule, the thickness of the lipid bilayer, the orientational order and dynamics of the lipid molecules, etc., seem to continue to change in response to increasing cholesterol concentrations at rates at least broadly similar in both the lower and higher cholesterol level regimes (see Refs. [7–11]]. In this regard, there are a number of studies indicating that the organization of the host phospholipid bilayer, and the orientation and depth of penetration of the cholesterol molecule, change continuously with cholesterol concentration up to levels of 50 mol.% (see Ref. [10]). Although it is not surprising that the properties of a single discrete thermodynamic phase change with the composition of that phase, the question of whether or not cholesterol/phospholipid

binary mixtures containing higher cholesterol levels are best described in terms of a single L_o phase with varying properties, the formation of various superlattices [36], or the formation of various molecular complexes [37], remains to be determined.

7. The differential interactions of cholesterol with phospholipids

Silvius has recently written an excellent review in which, among other things, he summarizes evidence accumulated over many years by a variety of physical techniques in different monolayer and bilayer model membrane systems relating to the preferential interaction of cholesterol with different classes and molecular species of glycerol- or sphingophospholipids [38•]. In general, such measurements show that the affinity of cholesterol varies significantly with the polar headgroup and backbone structure of the lipid molecule, generally decreasing in the order SM > PS > PC > PE. As well, in the glycerophospholipid PC, the affinity of cholesterol decreases markedly with an increase in the degree of unsaturation of the lipid chains. Of most relevance here is the value of the partition coefficient of cholesterol between egg PC (predominantly POPC) and brain SM, which favors the latter by approximately two-fold (see also Ref. [39]). This more favorable interaction between the saturated fatty acid-enriched natural SMs compared to the more unsaturated glycerophospholipids would seem to favor the formation of segregated cholesterol-depleted L_d and cholesterol-enriched L_o domains in bilayers composed of SM, PC and cholesterol, and thus provides support for the lipid raft hypothesis.

One must also consider, however, the stoichiometries of SM, glycerophospholipids and cholesterol in the plasma membranes of animal cells in considering whether or not the formation of cholesterol-depleted L_d and cholesterol-enriched L_o phases is possible in these membrane systems. For example, in the well studied human erythrocyte membrane, the major glycerophospholipids PC, PE and PS make up approximately 22.2, 19.6 and 8.9 mol.%, the phosphosphingolipid SM approximately 16.0 mol.%, and cholesterol approximately 33.3 mol.% of the total membrane lipids, neglecting the minor lipid components such as phosphatidylinositol and the various GSLs (see Ref. [2]). Thus, even considering the relatively higher affinity of cholesterol for SMs as compared to the various glycerophospholipids, most of the cholesterol in the human erythrocyte membrane will still not be bound to SM. In fact, even if the partition coefficient of cholesterol is many-fold higher for SM than for the total glycerophospholipid fraction, and even if SM and the glycerolipids were completely immiscible and formed entirely separate phases, then the amount of cholesterol in the glycerophospholipid phase would still exceed 25 mol.%. Assuming for the moment that the cholesterol/DPPC phase diagrams described above are generally applicable to other glycerol- and sphingophospholipids, as has been proposed, then both the relatively cholesterol-depleted glycerophospholipid domains and the relatively cholesterol-enriched SM domains would nevertheless both exist entirely or nearly entirely in the L_o phase. Since some mammalian plasma membranes contain lower amounts of SM and higher amounts of cholesterol, or both, relative to the human erythrocyte membrane, it is difficult to see how separate L_d and L_o phases as presently defined could coexist in such membrane systems. Moreover, a similar analysis of cholesterol/phospholipid stoichiometries and cholesterol relative affinities in lipid mixtures mimicking those of the inner and outer monolayers of the lipid bilayer of the human erythrocyte membrane, indicates that only the L_o phase should be present if cholesterol is evenly distributed in both leaflets of the bilayer. Moreover, even if the L_d phase would form, the L_o phase would certainly predominate and form the continuous phase within which smaller L_d phase domains would exist, in contrast to the predictions of the lipid raft hypothesis.

8. The in vivo evidence for lipid rafts

The limitations of the first type of in vivo evidence for the lipid raft hypothesis, namely, the existence of DRMs in animal or human cells, has been discussed earlier in this review and elsewhere [15•,16•,17] and will not be considered further here. Instead, we will briefly consider the other two pieces of evidence for lipid rafts, namely, the adverse effects of cholesterol depletion on membrane organization and function, and the existence of non-random lateral distribution of certain membrane proteins in the membrane plane. For a more detailed discussion of this material, see the three reviews referred to earlier [15•,16•,17].

A number of studies have reported that the presence of a certain amount of cholesterol is required for the isolation of DRMs from animal cells and for the optimal function of a number of protein-mediated plasma membrane processes. In these studies cholesterol levels are usually depleted by growing cells in cholesterol-deficient media or by reducing normal cholesterol levels in plasma membranes by treatment with cholesterol oxidase or by various cyclodextrins. Leaving aside for the moment the known perturbations of membrane lipid biosynthesis and turnover, and in lipid signaling invoked by these techniques, acute cholesterol depletion is also known to disrupt endocytosis and membrane trafficking, and to indirectly alter the organization of the cytoskeleton and its interaction with membrane proteins, among other processes. Moreover, cholesterol is well known to be essential for cell growth and to perform a variety of specific roles in membranes and cells, ranging from a

general regulation of the physical properties of the membrane lipid bilayer to specific interactions with membrane proteins, as well as functioning in metabolic regulation and signal transduction and other membrane-related processes. Since many of the cellular functions of cholesterol are either not known to, or not expected to, involve lipid raft formation, the disruptive effects of cholesterol depletion do not provide strong support for the raft hypothesis. A similar argument applies to the smaller number of studies in which sphingolipids have been depleted from the plasma membranes of animal cells. In this regard, one should note that studies in various prokaryotic microorganisms show clearly that the depletion of particular glycerophospholipids or glyceroglycolipids disrupt a number of membrane-associated processes, although the cell membranes of these microorganisms typically do not contain either cholesterol or sphingolipids and so can presumably not form either L_o phases or lipid rafts (see Refs. [40–44]).

A variety of studies have addressed the question of whether or not certain protein molecules associated with DRMs are clustered in the plasma membranes of animal or human cells. The results of studies using fluorescent microscopy generally show a uniform lateral distribution of labeled membrane proteins and lipid analogs in the membrane plane, suggesting either that lipid rafts do not exist or are too small to visualize. Other studies, utilizing fluorescence energy transfer or single-particle tracking to determine if lateral distribution or lateral diffusion of putative raft components, have yielded inconsistent results. Some studies have reported that both protein and lipid reporters exhibit no detectable clustering but seem to be randomly distributed, while other studies found evidence for local aggregation or confinement but report widely varying values for the diameters of the raft domains. Of course a major problem with all such studies, leaving aside technical issues of sensitivity and resolution, is that the lateral aggregation or the reduction of lateral diffusion of membrane proteins can also occur via specific interactions with other membrane proteins and with other cellular components such as the cytoskeletal network. Thus, even when the clustering of putative raft components is observed, this represents at best only weak evidence of the existence of lipid rafts in cell membranes. Moreover, the most sophisticated recent measurements seem to suggest that if lipid rafts exist, they are small in size (<10 nm) and persist for very short periods of time (approx. 1 ms or less) [43]. In fact, it has been suggested that these rafts may consist of only a few molecules of protein and associated lipids unless induced to form larger functional domains when their constituent protein are ligated [43–45]. If 'lipid rafts' in actual plasma membranes really are this small and unstable, one must question whether they are best described by the original raft hypothesis or simply as small protein oligomers associated with a shell of boundary or annulus lipid.

9. Conclusions

In this review, we have critically analyzed some of the major pieces of evidence which have been put forth in support of the lipid raft hypothesis. We do not find this evidence to be compelling or even internally consistent in some cases. We conclude that although the question of whether lipid microdomains exist in cell membranes is certainly an open one, a convincing case for their existence has not yet been made. Moreover, considering also the results of studies on biological as well as lipid model membranes (see Refs. [15•,16•,17]), we further conclude that the existence of relatively large and long-lived lipid raft domains in animal plasma membranes is unlikely.

Acknowledgments

We thank Drs Heiko Heerklotz and John R. Silvius for their valuable comments on an earlier draft of this review, and Dr Linda Pike for permission to reproduce Fig 3. Work from the senior author's laboratory was supported by operating and major equipment grants from the Canadian Institutes for Health Research, and major equipment grants from the Alberta Heritage Foundation for Medical Research. T.P.W. McMullen was a Teagle Scholar and a recipient of a Studentship from the Alberta Heritage Foundation for Medical Research.

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• of special interest
•• of outstanding interest

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