The 'double lives' of membrane lipids

Workshop: Anno 2000. A lipid milestone

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Physical properties of lipids 'shape' biomembranes

Membrane lipids like phosphatidylcholine (PC; see Table 1) spontaneously form bilayers in water. The behavior of such lipid assemblies is governed by the chemical structures of the lipids present. One characteristic property is the 'fluidity', which depends on the melting transition temperature of the lipids. The fact that many organisms regulate the fluidity of their membranes by adapting their lipid composition to the growth temperature demonstrates the importance of this parameter to membrane function. Among the highlights of the workshop was the presentation of data on the first membrane protein to be shown to act as 'cold' sensor, regulating membrane fluidity via signal transduction pathways (N. Murata, Okazaki, Japan; see section on signaling below). A second topic of high biological relevance was the fluid–fluid immiscibility of lipids. This refers to the coexistence of fluid domains with different properties, as occurs in mammalian cells, in contrast to the lipid phase separation into fluid liquid–crystalline and solid gel domains that has been well-characterized in model membranes and prokaryotes.

Cholesterol appears to be one of the key players in the formation of so-called liquid-ordered domains, for which various roles have been proposed in biomembranes, e.g. as platforms for protein and lipid transport and for cell signaling (Simons and Toomre, 2000). The rules underlying the formation of such domains are now being extensively studied in model systems. Gorter and Grendel (1925) showed that lipid monolayers are good for measuring molecular surface area versus lateral pressure (Figure 1). Using a monolayer set-up to determine phase characteristics of lipid mixtures, H. McConnell (Stanford, CA) has shown that condensed complexes are formed between cholesterol and certain phospholipids like sphingomyelin (Radhakrishnan *et al.*, 2000), 'complexes' in this context

Introduction

The year 2000 marks the 75th anniversary of the existence of the bilayer model of biological membranes as originally proposed by Gorter and Grendel (1925). It also marks a period of excitement in understanding membrane lipids. Many lipids appear to lead double lives, serving both as membrane building blocks and as modulators of important cellular processes. Membrane lipid behavior is becoming understood at molecular and even atomic levels, through the application of sophisticated biophysical and computational approaches. The molecular causes of membrane lipid-based diseases are being established, and new technologies whereby membranes and their components can be manipulated are now being used in medical and industrial applications. These topics were discussed at 'Anno 2000. A lipid milestone', and the highlights of this discussion are summarized below.

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Fig. 1. Monolayer set-up as used by Gorter and Grendel (1925). A lipid extract of erythrocytes was spread at the lipid–water interface in a Langmuir trough, after which the lipids were compressed by a floating barrier. The point at which the surface layer offered resistance was interpreted as the point at which a closely packed monolayer was formed. This area was twice the calculated surface area of the erythrocytes from which the lipids had been extracted, which led to the first lipid bilayer model.

implying a defined stoichiometry (as in cholesterol superlattice models). Tagging the ganglioside GM1 with fluorescently labeled cholera toxin B and observing its localization within membranes harboring such complexes revealed that it is found exclusively in the condensed complex-rich phase. Also, within intact, biological membranes, GM1 was seen to be limited to specific domains, suggesting that these domains represent condensed complexes.

One criterion for the existence of biomembrane domains is their resistance to extraction by detergent at low temperatures. J. Seelig (Basel, Switzerland) discussed the use of isothermal titration calorimetry (ITC) to analyze detergent–membrane interactions. He found that the detergent/lipid ratio at which a PC bilayer breaks up is linearly related to the product of the membrane partitioning coefficient of the detergent and its critical micelle concentration. Studies extending this technique to complex systems, whose components include cholesterol and sphingolipids, may help to determine the nature of the detergent resistance of lipid domains.

Biomembranes also harbor many lipids that, in isolation, tend to form non-lamellar, highly curved phases like cubic- or inverted hexagonal-phases. The balance between bilayer and such ‘non-bilayer’ lipids appears to be strictly regulated (de Kruijff, 1997). An obvious role for non-bilayer lipids would be to facilitate the formation of local, highly curved structures during processes like membrane fusion or vesicle budding. These bilayer destabilizing properties are currently being exploited in medical applications, to facilitate drug release from liposomes in cells (P. Cullis, Vancouver, Canada; see below). Non-bilayer lipids may also influence membrane protein activity or interactions with water-soluble components by affecting lipid packing properties and changing the lateral stress profile of the

membrane, i.e. the variation in lateral stress at different levels within the bilayer. Molecular dynamics simulations suggest that the stress profile may affect permeation of the bilayer by small molecules, since the levels at which both the lateral pressure (the opposite of stress) and the resistance to water diffusion are the highest coincide, residing just behind the lipid head groups (H. Berendsen, Groningen, The Netherlands).

General rules underlying protein–lipid interactions

Interactions of proteins with membranes are often governed by the general properties of the molecules involved. For charged proteins and lipids, electrostatic interactions are usually dominant. As an example of this, J. Seelig used ITC to show that the net apparent charge of the basic antibiotic peptide nisin is different in the presence of zwitterionic and anionic lipids. This finding suggests that, in anionic lipids, the positively charged part of the peptide interacts with the membrane surface first, while in zwitterionic lipids the hydrophobic part does so. S. McLaughlin (Stony Brook, NY) measured the binding of the myristoylated alanine-rich C kinase substrate (MARCKS) protein, and of small positively charged peptides corresponding to its basic effector domain, to lipid model systems. Results obtained using several techniques suggest that the basic effector region of MARCKS binds with high affinity to the polyvalent acidic lipid phosphatidylinositol bisphosphate (PIP₂), which is involved in several aspects of signal transduction.

A curious phenomenon of membrane biology is the promotion of secondary structure by protein binding to the bilayer surface. This occurs because partitioning of peptide bonds at the membrane–water interface is energetically costly. The relationship between binding and folding can be studied by using peptide analogs containing D amino acids, which interfere with folding but do not change the main physico-chemical properties of the peptides. This approach was taken by Seelig, using magainin, and by S. White (Irvine, CA) using melittin. Seelig found that the thermodynamic parameters of binding are linearly related to helicity, and that helix formation is a cooperative process that accounts for ~50% of the free energy of binding. White used small, unfolded peptides with different guest residues incorporated at specific positions, and compared the partitioning of these peptides in octanol and in bilayers. This resulted in the development of an interface scale, which showed that tryptophan (Trp) has a strong preference for the interface. Trp-containing peptides partitioned with a higher affinity in PC and phosphatidylserine than in phosphatidylglycerol (approximately –7 versus approximately –6 kcal/mole). Since the former two lipids contain a positive charge, this could be a first indication that favorable interactions between cations and pi-electrons in aromatic rings, as reported *in vacuo*, may affect protein–lipid interactions in membranes. White also presented a new experimental scale for predicting transmembrane regions of proteins with an extremely high accuracy (97%). This scale was based on the hypothesis that, because octanol can form hydrogen-bonds with the peptide backbone, partitioning of unfolded proteins in octanol is similar to partitioning of folded, intramolecularly hydrogen-bonded proteins in the membrane.

Although protein–lipid interactions can often be explained by the general properties of these molecules, many proteins appear to require interaction with a specific lipid to ensure proper folding, insertion or functional activity (Bogdanov and Dowhan, 1999). One striking example was given by W. Dowhan (Houston, TX), who showed that phosphatidylethanolamine (PE) is essential for proper folding of lactose permease (LacY) in the *Escherichia coli* inner membrane. In the absence of PE, the N-terminal half of the protein does not insert correctly, but the C-terminal half does, allowing facilitated, but not active, transport of lactose. Since the protein remains in its native state when the lipid is removed, PE was proposed to function as a ‘lipochaperone’. The molecular basis for the specific action of particular lipid molecules such as this one is not yet known.

Lipid transport and domains

Most lipids are synthesized on the cytosolic membrane surface. By what mechanism do the lipids partition between the two leaflets of cellular membranes? Although, in principle, the structure of the ER membrane (and bacterial membranes) may allow rapid lipid translocation, reconstitution studies suggest that the process is protein-mediated (A. Menon, Madison, WI). Many groups are trying to identify the aminophospholipid translocase, an ATP-driven lipid transporter whose activity was first observed 15 years ago. Surprisingly, a whole new class of lipid translocators has recently been found amongst the ATP-binding cassette (ABC) transporters, with potential substrates ranging from small hydrophobic drugs, bile acids and fatty acyl-CoAs to cholesterol, PC and glycosphingolipids. C. Raetz (Durham, NC) described the *E. coli* ABC-transporter MsbA, a 6-transmembrane domain protein involved in the synthesis of lipid A (endotoxin), the lipid anchor of lipopolysaccharide (LPS) on the external surface of the outer membrane. Lipid A synthesis is blocked in the inner membrane in the absence of MsbA, and mutants in helices 5 and 6 accumulated 95% of all newly synthesized [³²P]lipids in the inner membrane, including PE and phosphatidylglycerol. This suggests that MsbA plays a role in lipid synthesis by acting as a lipid flippase or, alternatively, by extruding lipids from the inner to the outer membrane.

What mechanisms ensure that the various cellular membranes retain their unique lipid compositions? The most striking differences in lipid composition are found between the ER and the plasma membrane, the plasma membrane being 10- to 20-fold enriched in sphingolipids and cholesterol. Some time ago, it had been suggested that sphingolipids and cholesterol are preferentially incorporated into the vesicles that move forward in the secretory pathway. Later, it was shown that sphingolipids, cholesterol and specific (GPI-anchored) proteins are relatively insoluble in detergent during transport, providing a first experimental indication for colocalization of these components in a membrane domain. Using detergent-insolubility as a criterion, sphingolipid/cholesterol domains were also found in the plasma membrane. Independently, the existence of these entities has been inferred from the characterization of the lateral diffusion of lipids and proteins on the cell surface. In order to understand the function of such domains at the molecular level, it would be important to have information about their sizes and half-lives. In tracings of single fluorescent molecules within muscle cell

Fig. 2. Sphingolipid/cholesterol rafts make thick membranes. A domain (orange) of tall sphingolipids (long thin green barrels) and cholesterol (short green barrel) surrounded by stocky unsaturated PC (thick green barrels in blue environment) in the outer monolayer, potentially complemented by phosphatidylserine/cholesterol rafts (yellow) in PE (green) on the cytosolic side of a mammalian plasma membrane. The depicted domain contains ~700 lipid molecules in each leaflet and has a thickness of ~50 Å versus a thickness of ~40 Å for the bulk membrane.

membranes (Schütz *et al.*, 2000), the lipid analog Cy5-diC14:0-PE, but not Cy5-diC18:1-PE (assumed to represent lipids preferring areas of lower and higher fluidity, respectively), was observed to be 100-fold enriched in domains of ~0.7 µm that were stable for several minutes (H. Schindler, Linz, Austria). Furthermore, when scanned over the surface of hepatoma cells by laser tweezers, beads attached to a GPI-protein (MHC class I), but not to di16:0-PE, experience non-elastic diffusion barriers of ~100 nm (areas of higher resistance of 100 nm diameter) that are independent of the actin skeleton and occur with the same frequency as caveolae (M. Sheetz, New York, NY).

The newest measurements of these domains, using single particle tracking, suggest that they are generally small, with a diameter of 50 nm (~3500 lipids), exist for >1 min, and comprise some 10 proteins. Given their size and the fact that they ‘carry’ proteins, these lipid domains have been termed ‘rafts’ (K. Simons, Heidelberg, Germany). It is assumed that rafts can be induced to coalesce by particular types of proteins, and the removal of cholesterol by cyclodextrin has been shown to abolish them. Interestingly, clustering of a typical raft GPI-anchored protein on the outside of the plasma membrane results in coclustering of an acylated protein on the cytosolic surface, illustrating that domains on the two surfaces are in some way coupled. This property appears to be fundamental to raft-mediated signaling (Figure 2). In monolayers, cholesterol was also found to form complexes with both sphingomyelin and phosphatidylserine, suggesting the occurrence of phosphatidylserine/cholesterol rafts on the cytosolic surface of membranes (McConnell; Radhakrishnan *et al.*, 2000). It is unclear how the rafts on opposite sides of the bilayer are connected, and how they are recognized by the vesicle budding machinery. Interestingly, the raft lipid galactosylceramide (GalCer) appears to act as a receptor for the HIV proteins gp120 and gp41 on the apical surface of intestinal cells, and to transport HIV across the epithelium in a cholesterol-dependent fashion, suggesting that rafts also function in the transcytotic pathway (M. Bomsel, Paris, France).

Membrane physics and signaling

One beautiful example of how the physical state of bio-membranes can be regulated by sensors, signal transduction and gene regulation is the lipid desaturase system in plants and cyanobacteria (referred to earlier in this review as a temperature sensor; Murata). A temperature shift from 34 to 22°C, and also hydrogenation of lipids of the plasma membrane at 36°C, enhances the expression of the gene for the acyl-lipid desaturase, whose substrates are the C1 fatty acyls of diacylglycerol lipids, within a matter of minutes. The sensor that enhances the gene expression, the histidine kinase Hik33, spans the membrane twice and phosphorylates the soluble histidine kinase Hik19. This kinase then switches on independent response regulators that bind to DNA and regulate transcription. A second level at which biomembranes can be regulated is through cholesterol synthesis. This regulation is mediated by a transcription factor released from the ER, the sterol regulatory element-binding protein (SREBP), by proteolysis in the Golgi. Cholesterol blocks ER exit, decreases transcription, and achieves feedback control of synthesis. The ER cholesterol concentration appears to depend on the chemical activity of cholesterol in the plasma membrane, i.e. its release rate, which is determined by condensed complex formation (McConnell).

The physical state of the membrane may also regulate physical cellular processes such as the rate of endocytosis. Sheetz provided a number of lines of evidence for this. For example, a 3-fold increase in the membrane tension during mitosis, as measured by pulling membrane tethers using optical tweezers, strongly reduced endocytosis, whereas an artificial reduction of the tension by detergent restored endocytosis. Furthermore, the membrane lipid PIP₂ was shown to regulate the balance between endo- and exocytosis by determining membrane tension via attachment to the actin skeleton. This was shown by experiments in which the addition of chlorpromazine to mouse 3T3 fibroblasts, or their transfection with the PH-domain of phospholipase C, both of which compete with actin for PIP₂ binding, reduced attachment and increased endocytosis. In yeast, the chemical structure or concentration of sterol in the plasma membrane affects endocytosis, most likely via a change in membrane structure. However, the effect of the sphingoid bases sphinganine and phytosphingosine appears to be through the actin cytoskeleton, via yeast casein kinase, or PKH plus PKC, yeast homologs of the mammalian phosphoinositide-dependent kinase and protein kinase C zeta, respectively (H. Riezman, Basel, Switzerland). In mammals, most protein kinase Cs are activated by diacylglycerol produced from PIP₂. The availability of PIP₂ for hydrolysis by phospholipase C and for actin-binding may be regulated by the PIP₂-binding MARCKS protein (McLaughlin). PIP₂ itself is synthesized by a family of PIP kinases whose localization appears to be regulated by the activity of casein kinase 2. Phosphorylated PIP kinase II α localizes to the plasma membrane, whereas PIP kinase II β resides in the nucleus (R. Irvine, Cambridge, UK).

Disease...

Given the importance of lipids to the integrity and function of cellular membranes, it would be surprising if defects in lipid

metabolism were not the cause of disease. It is already known that the phosphosphingolipid sphingomyelin and the single sugar-containing glycosphingolipids, glucosylceramide (GlcCer) and GalCer, are essential for mammalian survival. It has now become clear that more complex glycolipids are also essential. Half of the GM3-only mice, which can synthesize the simple ganglioside GM3 (sialic acid–galactose–GlcCer) but are unable to make the more complex gangliosides due to a double gene knock-out for the transferases that add to GM3 a second sialic acid or an *N*-acetylgalactosamine, die within 13 weeks (Kawai *et al.*, 2001). The lack of either a hydrolytic enzyme or activator (e.g. saposin) results in the development of a fatal sphingolipid storage disease, due to the accumulation of sphingolipids in the lysosomes of a number of cell types including neurons. In the most severe case, newborn mice lacking glucocerebrosidase are unable to hydrolyze GlcCer to ceramide in the skin, resulting in a fatal increase in water permeability (K. Sandhoff, Bonn, Germany). It is to be expected that, in future, milder diseases caused by defects in later stages of sphingolipid synthesis will also be identified.

In some mammalian organs, like brain and muscle, 50% of PE carries a long-chain alk-1'-enyl ether on the glycerol C1. This defect only marginally affects the physico-chemical properties of PE. However, a patient who lacks ether lipids due to a deficiency in the peroxisomal enzyme alkyl-DHAP synthase displays the clinical symptoms of rhizomelic chondrodysplasia punctata (RCDP). This particular illness is one of a series of fatal diseases (including Zellweger syndrome) in which severe growth- and mental retardation are caused by defective peroxisomal protein import and, thereby, lack of ether lipid synthesis (H. van den Bosch, Utrecht, The Netherlands). The elucidation of the exact function of ether lipids within cellular membranes remains a challenge.

...and cure

A number of current therapies for lipid-based diseases are based on recent insights into their biochemical natures. One example is the use of glycosphingolipid synthesis inhibitors for the treatment of sphingolipid storage diseases (Sandhoff), where they function by reducing substrate levels. In some cases, knowledge of the structures of lipids and of their inhibitors has made the development of drugs possible. In the case of obesity, drugs have been designed that function by binding covalently to the active center of pancreatic lipase in the gut (R. Verger, Marseille, France). Farnesyltransferase inhibitors (FTI), which are being used as anti-cancer drugs, function by preventing the prenylation of Ras, a modification that is required for its oncogenicity. Promising results also have been obtained with tetrapeptide inhibitors of the CaaX protease, the next protein in the prenylation cascade (P. Casey, Durham, NC). Finally, the solution of the crystal structure of one enzyme in *E. coli* lipid A synthesis has made possible the design of inhibitors that have proven to be extremely effective against the 'septic shock' that can be caused by the potent (pM) immunostimulatory activity of lipid A (endotoxin) in the blood. Progress in this particular area may ultimately lead to the design of mutants in lipid A synthesis that can yield surface-modified *E. coli* that can be used as adjuvants or vaccines (Raetz).

meeting report

Beyond the generation of disease inhibitors described above, knowledge of the properties of lipids in pure lipid model membranes has also led to the development of liposomes as highly efficient drug delivery systems. With bilayer pH gradients that provide low internal pH, drugs that are weak bases can be translocated across the bilayer and trapped inside at very high concentrations, even exceeding the solubility limit, with an efficiency of >99%, and within only a few minutes. Delivery to the cell can be further increased by using cationic lipid vesicles. On their own, cationic and anionic lipids can each form stable bilayers, but when mixed together they form inverted hexagonal phases, lipid cylinders with the polar headgroups pointing inwards. When such cationic vesicles, filled for instance with DNA required for gene therapy, are taken up by endosomes containing anionic lipids, the endosomal membranes are destabilized and the DNA is released (Cullis).

Next to drug design and delivery methods, as described above, rapid screening techniques form another essential component for the development of drugs that target membrane components. A variety of biosensors has already been developed using typical drug targets like G-protein coupled receptors and ion channels as models. For example, tethered bilayers carrying immobilized G-protein coupled receptors can be used for monitoring binding of ligands or drugs, and to follow G-protein activation and receptor deactivation via surface plasmon resonance. Also, membranes fused over small holes on chips can be used to monitor the activity of single channels, serving as an alternative to patch-clamp techniques, and allowing screens for drugs that interfere directly or indirectly with channel activity (H. Vogel, Lausanne, Switzerland).

Perspectives

The meeting described here illustrated how our understanding of the behavior of lipids, their biogenesis, and their various roles in cellular processes is rapidly growing, due to the development of new technologies and the application of genetic approaches. Single molecules can now be studied in intact cells with a variety of techniques, and more advanced biophysical techniques are being used to investigate complex systems. Computational approaches are gaining in importance due to increasing computer power, and simulation of relatively slow processes, such as domain formation, membrane fusion or transport of lipids across membranes is becoming feasible. As more specific interactions between individual molecules within cells are established, it will become increasingly important to identify their molecular bases. And, as more and more information becomes available, a big challenge will be to determine the significance of experimental observations in the context of the living cell.

Table I. Biomembrane terminology

Abbreviation	Term
ABC	ATP-binding cassette
FTI	farnesyltransferase inhibitors
GalCer	galactosylceramide
GlcCer	glucosylceramide
ITC	isothermal titration calorimetry
LPS	lipopolysaccharide
MARCKS	myristoylated alanine-rich C kinase substrate
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol

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