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MEMBRANE INTERACTIONS OF SMALL SOLUTES

Studies with drugs and osmolytes

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ABBREVIATIONS

brainPS, phosphatidylserine from bovine brain

CLZ, clozapine

CPZ, chlorpromazine

CsA, cyclosporin A

DMPC, 1,2,-myristoyl-*sn*-glycero-3-phosphocholine

DPPC, 1,2-palmitoyl-*sn*-glycero-3-phosphocholine

DSC, differential scanning calorimetry

eggPC, egg yolk phosphatidylcholine

GP, Laurdan emission generalized polarization

HPD, haloperidol

I_e, intensity of the pyrene excimer emission

I_m, intensity of the pyrene monomer emission

I_e/I_m, pyrene excimer-to-monomer ratio

Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene

LUV, large unilamellar vesicle

MLV, multilamellar vesicle

NBD-PC, 1-palmitoyl-2-(N-4-nitrobenz-2-oxa-1,3-diazol)aminocaproyl phosphocholine

PC, phosphatidylcholine

PE, phosphatidylethanolamine

PG, phosphatidylglycerol

PgP, P-glycoprotein

POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol

PPDPC,1-palmitoyl-2-[(6-pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine

PPDPG, 1-palmitoyl-2-[(6-pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphoglycerol

RA, relative amplitude

RET, resonance energy transfer

T_m, main transition temperature

T_p, pretransition temperature

$t_{1/2}$, halftime of the fluorescence decay

X, mole fraction of the indicated compound

γ, surface tension

π, surface pressure

π_0, initial surface pressure

$\Delta\pi$, change in surface pressure

ΔH_m, enthalpy of main transition

ΔH_p, enthalpy of the pretransition

Ψ, membrane dipole potential

Π, osmotic pressure

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals I-IV.

I. Tim Söderlund, Arimatti Jutila, and Paavo K.J. Kinnunen. 1999. Binding of adriamycin to liposomes as a probe to membrane lateral organization. *Biophysical Journal*, 76: 896-907.

II. Tim Söderlund, Jukka Y.A. Lehtonen, and Paavo K.J. Kinnunen. 1999. Interaction of cyclosporin A with phospholipid membranes. Effect of cholesterol. *Molecular Pharmacology,* 55: 32-38.

III. Arimatti Jutila,Tim Söderlund, Antti Pakkanen, Matti Huttunen, and Paavo K.J. Kinnunen. 2001. Comparison of the effects of clozapine, chlorpromazine, and haloperidol on membrane lateral heterogeneity. *Chemistry and Physics of Lipids*, 112: 151-163.

IV. Tim Söderlund, Juha-Matti I. Alakoskela, Antti L. Pakkanen, and Paavo K.J. Kinnunen. Comparison of the effect of surface tension and osmotic pressure on the interfacial hydration of fluid phospholipid bilayer. *In revision*.

Publication III has been used previously as a part of Ph.D. Arimatti Jutila´s dissertation ”Lateral heterogeneity in model membranes – inducements and effects” 2001, Medical Faculty, University of Helsinki.

SUMMARY

Lipids form the main structural element of cellular membranes. The lack of covalent interactions between lipids within these assemblies provides the basis for the highly dynamic properties of biological membranes. Lipids are synthesized by complex enzymatic machineries, and a living cell contains a several hundred different lipid species. The lateral organization of the membranes affects the functions of proteins. The plasma membrane provides a permeability barrier for the cell and defines its outer boundaries. Lipid membranes also form intracellular compartments with specialized (colocalized) functions.

Proteins and DNA are well established targets for medical intervention (drugs) but much less is known about the importance of lipids as drug targets. Drug-lipid interactions are important, as the absorption and distribution of drugs at the level of cells and organisms are highly dependent on the membrane permeability of these compounds.

Membrane propeties affecting the drug-lipid interactions were studied in this thesis, as well as changes in membrane properties due to the membrane association of the drugs. A new finding was that membrane lateral heterogeneity can have large impact on the membrane association of a model drug, doxorubicin. Also, novel effect of drugs on the membrane was demonstrated as several drugs were shown to alter the membrane lipid domain morphology in lipid monolayers. In addition, the effect of cholesterol on the lipid interactions of Cyclosporin A were studied, and Cyclosporin A was observed to have strong influence on cholesterol containing membranes. Drug-lipid interactions of three neuroleptic drugs (clozapine, chlorpromazine, and haloperidol) were investigated. Clozapine was observed to have a different mode of membrane interaction as compared to the two other neuroleptics. This is of interest, as clozapine shows clearly different effects in clinical use.

The factors modulating membrane properties are important in drug-lipid interactions. Therefore, the effect of surface tension and osmotic pressure on the interfacial dynamics of a phospholipid bilayer were also compared. Three inert solutes, viz. betaine, choline chloride, and sucrose were used. The effects on the interfacial

dynamics correlated with the increment of surface tension while no correlation to osmotic pressure was observed. These results are readily explained in the framework of membrane lateral pressure profile, i.e. surface tension exerts its effects on the thin interfacial region of the lipid membrane, causing increased packing. These findings suggest a surface tension mediated mechanism for controlling the membrane organization and structure.

1. REVIEW OF THE LITERATURE

In the following sections, the structure and function of model and biomembranes are briefly outlined. The importance of the drug-lipid interactions are also introduced with some examples.

1.1. BIOLOGICAL MEMBRANES

Structure and composition

Lipid bilayers form the structural core of cellular membranes. Lipid bilayers in biological membranes are composed of hundreds of different lipid species (Myher et al., 1989). The plasma membrane forms the barrier between the extra- and intraccellular milieu and intracellular membranes colocalize certain functions into different cellular organelles, for example, the oxidative phosphorylation in mitochondria, the degradation of protein, lipids, carbohydrates, and nucleic acids in lysosomes, and the posttranslational modification of proteins in the golgi apparatus (Lehninger et al., 1997). Biomembranes contain various amounts of integral and peripheral proteins, and each organelle has characteristic membrane phospholipid compositions (Lehninger et al., 1997). The physical state of the membrane also varies from organelle to organelle (Mamdouh et al., 1998). Cholesterol content is highest in the plasma membrane, and lowest in mitochondria, whereas the negatively charged cardiolipin containing four acyl chains, is mainly found in the inner mitochondrial membrane (Lehninger et al., 1997), and glycosphingolipids have an apical/basolateral polarity 17-fold higher than phosphatidylcholines (van Meer and van Genderer, 1994). Additionally, some lipid species are highly enriched in certain cell types, for example neurons have an absolute requirement for ω-3 fatty acid containing lipids in order to maintain their physiological function (Fliesler and Anderson, 1983; Salem and Ward, 1993; Salem and Niebylski, 1995). To this end, biomembranes are highly heterogeneous in composition and functions within and between cells.

Dynamics and organization

The fluid mosaic model presented by Singer and Nicolson (1972) describes biomembranes as a fluid matrix in which membrane proteins and lipids diffuse freely. The current view of biomembranes is different in many aspects (Mouritsen and

Kinnunen, 1996; Mouritsen and Andersen, 1998). It has become evident that membrane components do not diffuse freely in the plane of the membrane, as the diffusion is limited by multiple factors, such as the cytoskeleton and tight junctions (van Meer and van Genderer, 1994). A large body of evidence has accumulated on the structural organization of lipids in cellular membranes (Kinnunen, 1991; Kinnunen et al., 1994; Mouritsen and Kinnunen, 1996). In plasma membranes the distribution of lipids between intra- and extracellular leaflet is asymmetric and is maintaned by ATP dependent systems, resulting in enrichment of cholesterol and sphingolipids on the extracellular leaflet, and depletion of negatively charged phosphatidylserine from the extracellular leaflet (Op den Kamp, 1979; Lehninger et al., 1997; Tocanne et al., 1994). The transbilayer movement of phosphatidylserine to the extracellular side is important for the platelet aggregation in blood vessels (Bevers, et al., 1999), and is also one landmark of apoptosis (Adayev et al., 1998). Lateral organization of biomembranes has been suggested to be important for the physiological function (Welti and Glaser, 1994; Mouritsen and Kinnunen, 1996; Prenner et al., 2000; Schütz et al., 2000). Accordingly, changes in membrane lateral organization occur during cell cycle and sperm maturation (Welby et al., 1996; Sivashamagam and Rajalakshmi, 1997; Flesch and Gadella, 2000). Likewise, a decrease in membrane cholesterol content and microviscosity in hippocampal and cortex regions of rat brains has been observed to correlate with learning processess (Kessler and Yehuda, 1985). The metastatic potential of LM fibroblasts is highly correlated with the ratio of membrane fluidity of intracellular/extracellular leaflets of the plasma membrane (Schroeder et al., 1995). The membrane lipid composition also varies in high and low metastatic variants of human prostatic cancer cell line (Dahiya et al., 1992), acyl chain unsaturation in excised leiomyosarcoma correlates with the grade and mitotic activity of the cancer cells (Singer et al., 1996), and apoptotic thymocytes can be identified by their decreased membrane lateral packing (Schlegel et al., 1993). The membrane microviscosity at the hydrocarbon core of plasma membranes of endothelial cells is decreased in hypertensive subjects as compared to healthy subjects (Zicha et al., 1999). The physicochemical state of the biomembranes is important as shown by studies indicating tightly regulated control of membrane lipid composition in response to several stimuli such as increased growth temperature, altered fatty acid content of the diet, and exposure to drugs (Christiansson et al., 1985; Lehninger et al., 1997; Luxo et al., 1998). Several microorganisms maintain their membrane lipid

composition such that the phase state of the fluid membrane is below the lamellar-to-nonlamellar phase transition, but on the prevailing condition there is a tendency to form non-lamellar phases (Goldfine, 1984; Wieslander et al., 1986; Rietvield et al., 1994; Morein et al., 1996). There is also some evidence that both eukaryotic and prokaryotic cell membranes (at the growth temperature) are close to phase boundaries corresponding to lipid phase separations (Linden et al., 1973; Linden and Fox, 1975; Beehan-Martin et al., 1993)

1.2. LIPID DYNAMICS

Intra- and intermolecular dynamics

Lipid bilayers are highly dynamic structures and lipid dynamics can be divided into two categories: conformational and translational. The former describes the intramolecular motions, while the latter indicates the lateral position of the molecule in the plane of the membrane. The rate of the translational movements is much slower than those of intramolecular motions. **(Fig. 1)**

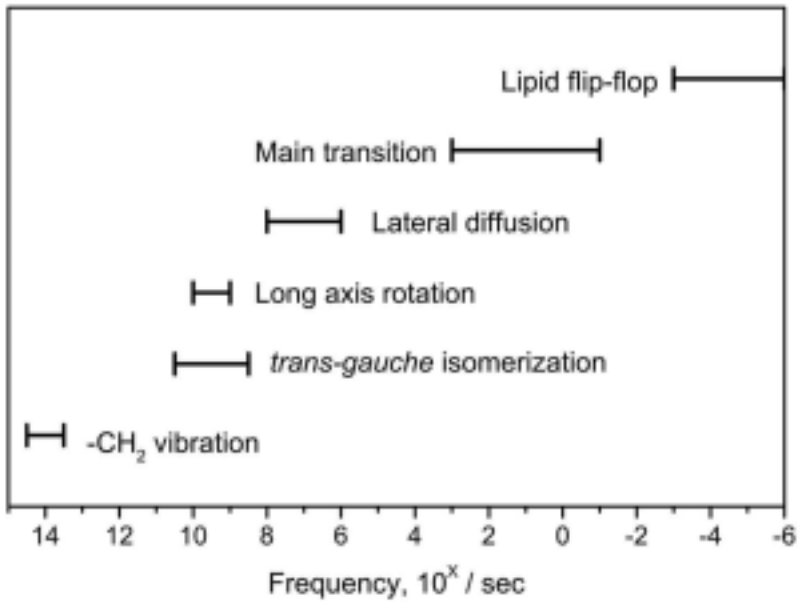


Figure 1. The characteristic frequencies of conformational and translational lipid dynamics in membranes (van Meer and Genderen, 1994).

The free volume of the membrane describes the difference between the effective and the van der Waals volumes per molecule (Bondi, 1954; Cohen and Turnbull, 1959; Turnbull and Cohen, 1970). In lipid bilayers, free volume arises from short-lived,

dynamic defects within the hydrocarbon core, due to *trans-gauche* isomerizations resulting from the packing constraints and thermal motion (Xiang, 1993). Membrane free volume is an important determinant for the lateral diffusion in the membranes (Galla et al., 1979). Lipid dynamics are highly dependent on many factors, such as the acyl chain length, number of *cis*-double bonds, lipid composition, pressure, degree of hydration, temperature, and phase state of the membrane (Kinnunen et al., 1994). The commonly used term ”membrane fluidity” is usually not well-defined, and it can be considered to contain contributions from both conformational (microviscosity) and translational (lateral diffusion) dynamics (Mouritsen and Kinnunen, 1996).

Lateral pressure profile

The distribution of the lateral pressure is not homogenous throughout the membrane. **(Fig. 2)** Thermal motions cause conformational disorder in the acyl chain region which leads to the tendency for lateral expansion. Binding of water to the lipid headgroups increases lateral repulsion between headgroups causing membrane expansion. The lateral expansion is balanced by the interfacial tension generated at the apolar (hydrocarbon) polar (water) interface due to the unfavourable water-hydrocarbon contacts (Seddon and Templer, 1995; Cantor, 1997a).

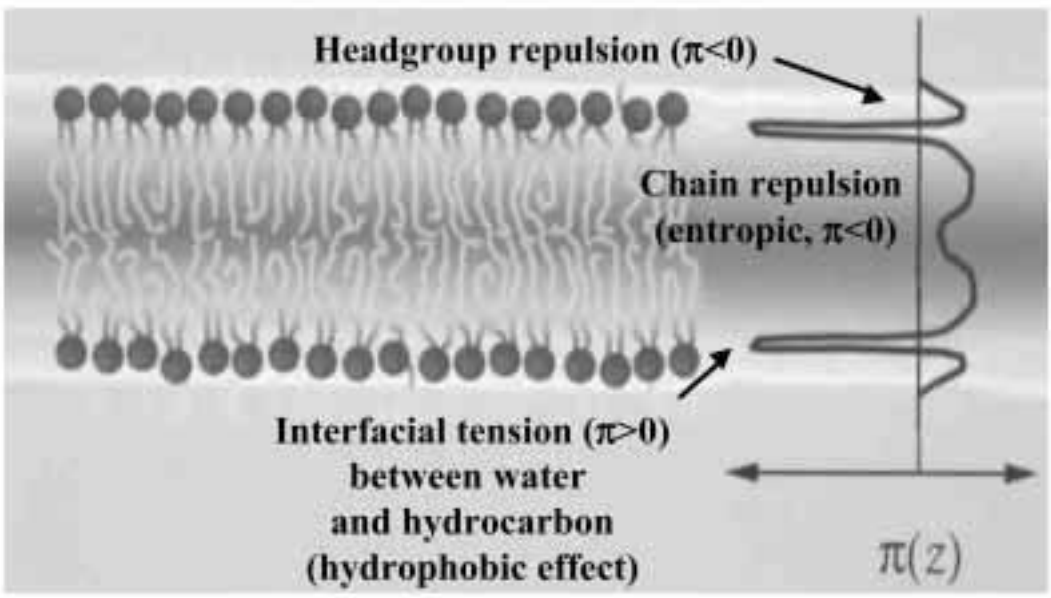


Figure 2. Membrane lateral pressure profile. Headgroup and chain repulsion are compensated by interfacial tension, as a results π=0 (Kinnunen, 2000).

This interfacial pressure can correspond to bulk pressures of many hundred atmospheres (Cantor, 1997a; Cantor, 1999a). The interfacial tension represents a direct expression of the hydrophobic effect (Tanford, 1980; Cevc and Marsh, 1987). In some cases there can be attractive potential between polar headgroups, due to the hydrogen bonding between headgroups (Boggs, 1987).

The equilibrium lateral pressure of lipid bilayers in model and biomembranes has been estimated to range from 20 mN/m to 56 mN/m (Demel et al., 1975; Fulford and Peel, 1980; Gruen and Wolfe, 1982; Jähnig, 1984; Nagle, 1986; Konttila et al., 1988; Marsh, 1996; Crane et al., 1999), but most commonly values of ~30-40 mN/m are believed to be in the correct magnitude (Marsh, 1996).

Lipid bilayer as a permeability barrier

The lateral pressure profile provides a framework for the description of the membrane as a permeability barrier. The major barrier for water permeability across lipid bilayer is formed by the first 7-8 carbons from the carbonyl ester groups (Inoue et al., 1985). These carbon atoms have the lowest degree of freedom within the bilayer (Petrache et al., 2000). Permeability through the lipid bilayer is dependent on the lipid packing both in the interfacial region as well as in the hydrocarbon core (Xiang and Anderson, 1997). The partitioning of benzene into membranes depends on the lateral packing of the phospholipid acyl chains. By increasing packing density from half maximal to 90 % of maximum, the partitioning of benzene decreases by an order of magnitude, and the partitioning process is not dependent on the nature of the agent used to modify the density, as temperature, cholesterol and acyl chain length cause the same effect (Young and Dill, 1988). Partitioning of compounds into interfacial phase, such as a bilayer is fundamentally different than partitioning between two bulk phases, such as octanol-water partition, which is widely used in drug characterization (Young and Dill, 1988).

1.3. MEMBRANE HYDRATION

In aqueous solution water molecules associate with lipid headgroups, and interfacial carboxy-ester groups. This ”bound water” forms the hydration shell of the lipid (Jendrasiak, 1996). The physical properties of the bound water differ from those of free, bulk water. The freezing point of bound water can be below 0 ^{o}C, and translational and rotational properties, electrical conductivity as well as density also differ from bulk water (Jendrasiak, 1996). The magnitude of these membrane effects on the properties of water decay exponentially as a function of distance from the surface, and the properties of water molecules in the hydration shell are not homogeneous (Jendrasiak, 1996). It should be emphasized that the hydration shell is dynamic, not static in nature.

The hydration of the hydrophilic headgroup plays an important role in the structure and function of phospholipid bilayers (Jendrasiak, 1996). The membrane surfaces have significant exposure of hydrophobic surface (Marrink and Berendsen, 1994). Penetration of water molecules into lipid bilayers is not homogeneous and although lipid headgroups are charged the organization of water molecules in the hydration shell of phosphatidylcholine (PC) has been proposed to be similar to an idealized clathrate structure of water around apolar solutes (Alper et al., 1993). Water associated with lipid headgroups and the interfacial region is the main determinant of the dipole potential of lipid membranes (Gawrisch et al., 1992; Brockman, 1994). The number of water molecules in the hydration shell depends on the phase state of lipids (McIntosh, 1996), the type of lipid headgroup (McIntosh, 1996), acyl chain composition and the presence of *cis*-double bonds (Jendrasiak and Hasty, 1974). The presence of compounds such as sterols increase membrane hydration (Jendrasiak and Mendible, 1976; Marsh, 2002), which is augmented as the distance between adjacent headgroups is increased by the additives (Jendrasiak and Hasty, 1974; Jendrasiak and Mendible, 1976). Dehydration of phospholipid membranes has been shown to induce phase separation (Webb et al., 1993; Lehtonen and Kinnunen, 1995) and lamellar-to-hexagonal-II phase transition (Webb et al., 1993). Dehydration of phospholipid membranes is also an essential prequisite to the fusion of lipid membranes (Wilschut and Hoekstra, 1986).

Any aqueous compartment (e.g. membrane surface) that is inaccessible to solute has its water activity controlled by the solute concentration, the activity being lowered by the addition of nonwater molecules (Parsegian and Rand, 1995; Parsegian et al., 2000; Rand et al., 2000). The exclusion of water from some regions can be obtained by two qualitatively different mechanisms. When a solute is too large to enter into the region, steric exclusion occurs (Parsegian et al., 2000; Rand et al., 2000). Preferential hydration means that the interface prefers to interact with water rather than with the solute (Parsegian et al., 2000; Rand et al., 2000). However, preferential hydration and steric exclusion can occur simultaneously in the same system. On the other hand, the solute may prefer to interact with water more strongly than with the interface. Under this osmotic pressure the components of the molecular assembly are pushed together (Parsegian and Rand, 1995).

The introduction of different solutes in an aqueous medium induces structural changes in water, and these compounds are described as "structure breakers" (chaotropes) or "structure makers" (kosmotropes), depending on their effect on the hydrogen bonded network of liquid water (Luu et al., 1990; Collins, 1997). Kosmotropes tend to decrease the interfacial area while chaotropes have the opposite effect. The magnitude of these changes depend also on the degree of solute depletion (kosmotropes) or enrichment (chaotropes) at the interfaces, in comparison to bulk phase (Koynova et al., 1998). Typically, compounds accumulating on the interface decrease surface tension.

1.4. MEMBRANE LATERAL HETEROGENEITY

The lateral organization in model and biomembranes occurs in various lengthscales from nanometer to micrometer scale (Sankaram et al., 1992; Mouritsen and Jørgensen, 1994; Tocanne et al., 1994; Hwang et al., 1995; Mouritsen and Kinnunen, 1996; Schram et al., 1996; Gliss et al., 1998; Schütz et al., 2000; Loura et al., 2001), leading to the formation of lipid domains, which are characterised as an area of membrane having different lipid compositions and/or physicochemical properties than the neighbouring regions (Welti and Glaser, 1994; Mouritsen and Kinnunen, 1996; Brown, 1998). Lipid domains have been observed in both model and biomembranes and the importance of these domains on the biological processes emphasizes the need

to understand the underlying physicochemical factors for domain formation and dynamics (Welti and Glaser, 1994; Mouritsen and Kinnunen, 1996; Mouritsen and Jørgensen, 1997; Stillwell et al., 2000; Leidy et al., 2001).

The coexistence of gel and fluid phases as well as fluid-fluid immiscibility have been demonstrated for binary phospholipid mixtures (Pagano et al., 1973; Shimshick and McConnell, 1973; Wu and McConnell, 1975; Mabrey and Sturtevant, 1976; Sankaram et al., 1992; Hinderliter et al., 1994; Klinger et al., 1994; Jørgensen and Mouritsen, 1995; Thompson et al., 1995; Schram et al., 1996; Holopainen et al., 1997; Gliss et al., 1998; Holopainen et al., 2000a; Leidy et al., 2001; Loura et al., 2001). In lipid membranes containing charged phospholipids, phase separation or microdomain formation can be induced by Ca^{2+} (Galla and Sackmann, 1975; Ashley and Brammer, 1984; Eklund et al., 1988), polycations (Galla and Sackmann, 1975; Eklund and Kinnunen, 1986), electric fields (Lee et al., 1994; Lee and McConnell, 1995), DNA (Kôiv et al., 1994), peripheral cationic proteins (Kinnunen et al., 1994), peptides (Hartmann et al., 1977; Denisov et al., 1998), and pH (Tilcock and Cullis, 1981). Changes in membrane lateral organization are also caused by interactions between integral membrane proteins and lipids (Sperotto and Mouritsen, 1993). Membrane dehydration was proposed to induce domain formation (Bryant and Wolfe, 1989) and has been experimentally verified for binary lipid mixtures (Lehtonen and Kinnunen, 1995). Lateral heterogeneity can also be induced by alcohol (Rowe, 1987; Mou et al., 1994), and by dynamic fluctuations at the main transition (Freire and Biltonen, 1978; Pedersen et al., 1996; Nielsen et al., 2000). Mismatch between the effective length of the phospholipid acyl chains can also cause the formation of microdomains (Lehtonen et al., 1996a). Mismatch between the hydrophobic thickness of phospholipids and integral membrane protein was first predicted to cause ordering of the membrane (Mouritsen and Bloom, 1984; 1993), and later experimentally demonstrated for bacteriorhodopsin (Piknová et al., 1993; Dumas et al., 1997) and lactose permease (Lehtonen and Kinnunen, 1997).

The regular distribution of lipids into hexagonal superlattices provides another mechanism for membrane organization. Based on the observed maximas/minimas on the fluorescence intensity, anisotropy, and lifetime measurements, and by theorethical calculations, fluorescent phospholipid analogs and sterols have been suggested to

distribute regularly in membranes, forming hexagonal superlattices (Somerharju et al., 1985; Kinnunen et al., 1987; Virtanen et al., 1988; Tang and Chong, 1992; Sugar et al., 1994; Chong et al., 1994; Tang et al., 1995; Cheng et al., 1997; Liu et al., 1997). The regular distribution has been suggested to originate from long-range repulsive interaction between the guest molecules (Sugar et al., 1994), as well as from the steric elastic strain (Sackmann, 1983), whereas rapid lateral diffusion can destroy the regular organization (Tang et al., 1995).

1.5. LIPID-PROTEIN INTERACTIONS

Lipid-protein interactions are not trivial modulators of protein function and cellular signalling cascades. The knowledge on lipid-protein interaction is also warranted for better understanding of the significance of drug-lipid interactions on the effects of drugs.

Several membrane properties have been observed to influence the function of peripheral and integral membrane proteins. Some proteins require specific lipid species in the membrane for proper and/or optimal activity (Srivastava et al., 1987). Phospholipase A_2 is perhaps the most studied peripheral membrane protein in respect to the effect of membrane lateral heterogeneity onto its activity. In brief, the interfacial boundary between lipid domains creates structural defects in the membrane, and the activity of phospholipase A_2 is increased in the presence of these defects (Mouritsen and Biltonen, 1993; Burack et al., 1993; Burack and Biltonen, 1994; Hønger et al., 1996; Huang et al., 1998). Likewise, phospholipase C shows an anomalous increase in its activity near the T_m (Holopainen et al., 2002). H_{II}-propensity is a tendency for formation of non-lamellar phases, without actual formation of such phases (Epand, 1991). The activity of several peripheral membrane proteins, such as protein kinase C (Slater et al., 1994) and CTP:phosphocholine cytidyltransferase (Epand, 1991; Jamil et al., 1993), and integral proteins, such as rhodopsin (Brown, 1994), alamethicin (Keller et al., 1993), and insulin receptor tyrosine kinase (McCallum and Epand, 1995) have all been shown to be snesitive to H_{II}-propensity.

Membrane lipid dynamics can affect the conformational state of integral proteins (Cantor, 1997b; 1999b), as well as the interactions between peripheral membrane proteins and membrane surfaces (Kinnunen et al., 1994). The depth of the changes in membrane dynamics can play a dominant role (Cantor, 1997a; 1997b; 1999a; 1999b), as shown for the opioid receptor, whose substrate binding was sensitive to changes in membrane interfacial microviscosity, but not to hydrocarbon microviscosity (Lazar and Medzihradsky, 1992). The lipid phase state also modulates the functions of integral membrane proteins, as shown for the lactose permease (Zhang and Kaback, 2000). Membrane attachment of chromosome replication initiation protein dnaA is required for its function. The fluidity of the lipid membrane is important for the function, and factors increasing fluidity, such as temperature, cis-double bonds, small amounts of cholesterol, or certain drugs enhance the activity of dnaA protein (Yung and Kornberg, 1988).

Hydrophobic mismatch between lipids and integral membrane proteins affect the membrane lateral organization but hydrophobic mismatch can also have large impact on the activity of proteins, as shown for the leucine transporter (In´t Veld et al., 1991), cytochrome c oxidase (Montecucco et al., 1982), (Na^+-K^+)-ATPase (Johansson et al., 1981a), and Ca^{2+}-ATPase (Caffrey and Feigenson, 1981; Johansson et al., 1981b: Cornea and Thomas, 1994).

1.6. PHASE TRANSITIONS

Phospholipids exist in two categories, namely thermotropic and lyotropic (Kinnunen and Laggner, 1991). Biological membranes are mainly in the fluid lamellar phase. Therefore, the main emphasis will be put on the gel and fluid phases and gel → fluid phase transitions. **(Fig. 3)** In the gel phase, the average cross-sectional area per hydrocarbon chain is approx. 20 $Å^2$ (Seddon and Templer, 1995). At pretransition the lipid molecules become tilted. This arrangement allows larger headgroup area per lipid molecule, and the pretransition is accompanied with increase in headgroup hydration (Janiak et al., 1979; Okamura et al., 1990; Cevc, 1991; Seddon and Templer, 1995). The lipid bilayer further expands in gel (L_α)→ fluid (L_β) transition (Seddon and Templer, 1995). The interfacial area per molecule increases by 15-30 %, with decreasing bilayer thickness, and onset of rapid lateral diffusion (Seddon and

Templer, 1995). The main transition temperature (T_m) of fully hydrated lipid bilayers is dependent on the headgroup, chain saturation, and acyl chain length (Koynova and Caffrey, 1998). T_m increases linearly with added carbon atoms, and decreases with increasing number of *cis*-double bonds (Koynova and Caffrey, 1998).

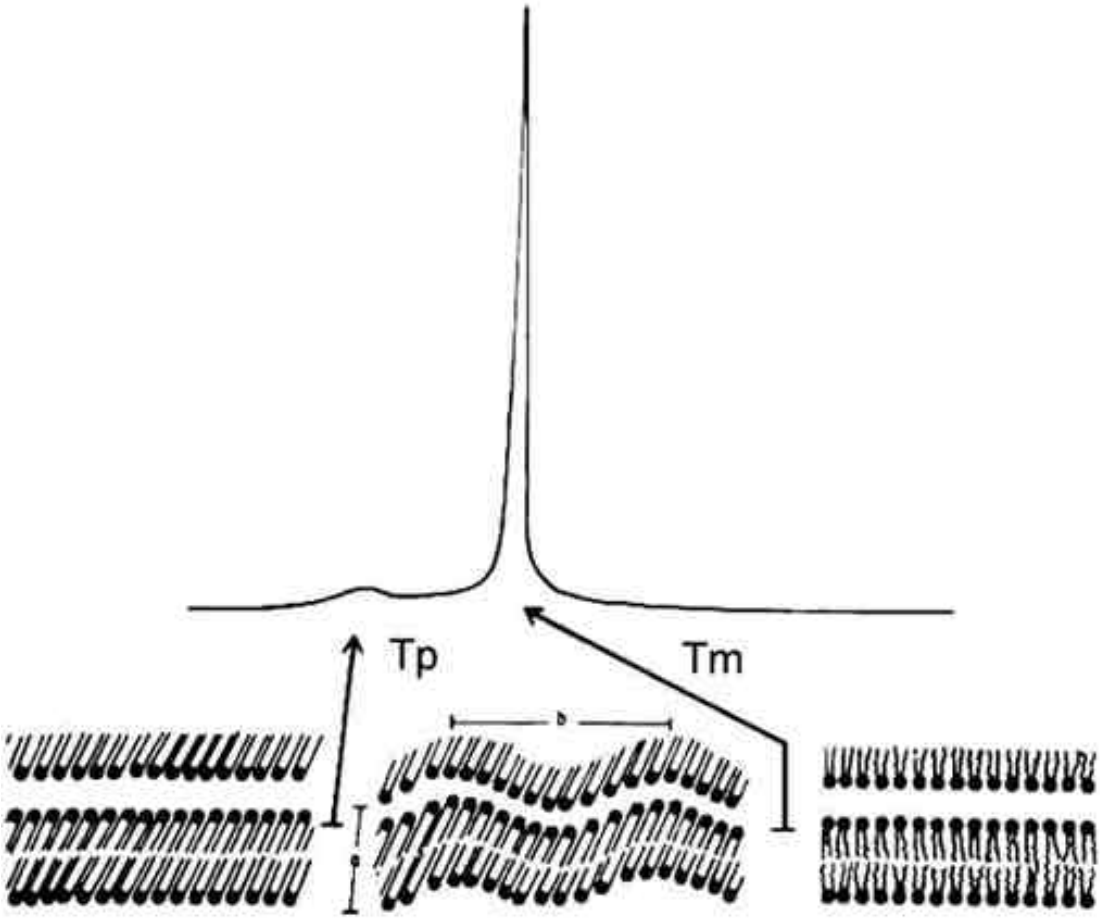


Figure 3. DSC endotherm for DPPC MLVs showing the pretransition (T_p) and main transition (T_m) peaks. Schematic representation of gel, rippled, and fluid phases (from left to right) are shown.

Dynamic lateral heterogeneity due to the coexisting gel and fluid domains in the main transition has been suggested (Marsh et al., 1977; Doniach, 1978; Freire and Biltonen, 1978; Pedersen et al., 1996; Jutila and Kinnunen, 1997). The fluctuations of the coexisting gel and fluid domains are likely causes of the observed maximum in heat capacity at T_m and maximums in lateral compressibility, bending elasticity, membrane permeability and activity of phospholipase A_2 (Papahadjopoulos et al., 1973; Op den Kamp et al., 1975; Marsh et al., 1976; Doniach, 1978; Freire and Biltonen, 1978; Nagle and Scott, 1978; Evans and Kwok, 1982; Maynard et al., 1985; Menashe et al.,

1986; Bloom et al., 1991; Hønger et al., 1997), and these effects have been attributed to the presence of domain boundaries (Papahadjopoulos et al., 1973; Hønger et al., 1996). The length of the interfacial boundary between gel and fluid domains has been calculated to have a maximum at T_m (Freire and Biltonen, 1978). Based on the pyrene fluorescence emission and resonance energy transfer measurements the main transition has been suggested to proceed from gel to fluid via an intermediate phase, characterized as a strongly fluctuating superlattice (Jutila and Kinnunen, 1997).

1.7. DRUG-LIPID INTERACTIONS

Why to study drug-lipid interactions?

Most of the currently used drugs are amphiphilic in nature, and have a tendency to self-associate and interact with membranes (Schreier et al., 2000). The main barrier for passive diffusion through cellular membranes is the lipid bilayer. The differences in lipid compositions affect the passive diffusion of compounds through lipid membranes, and eventually affect on the accumulation of drugs in cells (Burns and North, 1986; Escriba et al., 1990; Mason et al., 1992; Callaghan et al., 1993). Elucidation of the mechanisms affecting the passive diffusion of compounds through the lipid bilayer is of primary importance in drug development. Also, the overexpression of the P-glycoprotein (PgP) is one of the major causes for multidrug resistance in human cancers (Gottesman and Pastan, 1993; Bosch and Croop, 1996). PgP is an integral membrane protein, and the effective function of PgP requires the substrate to be located within the membrane (Romsicki and Sharom, 1999; Lu et al., 2001). Several drugs are known to be efficiently pumped out from the cell by PgP, and increase in the intracellular concentration of PgP substrates can be obtained by co-administrating PgP inhibitor and substrate (Bosch and Croop, 1996). Conventionally, drug-lipid interactions are neglected as a possible explanation for increased intracellular concentration of the substrate in studies of PgP function. However, a PgP inhibitor (verapamil), also increased the passive diffusion of a substrate (doxorubicin) through liposomal and cytoplasmic membranes (Drori et al., 1995; Speelmans et al., 1995). The drug-lipid interactions are playing a important role in physicochemical characterization of compounds during drug development, and passive membrane permeation is the main pathway for drugs to cross cellular barriers (Faller and Wohnsland, 2001).

Binding of drugs to membrane lipids can lead to alterations in the function of proteins as shown for phospholipase A_2 (Mustonen et al., 1991), cytochrome c oxidase (Nicolay and de kruijff, 1987), lysosomal phospholipases, phosphatidylinositol specific phospholipase C, sphingomyelinase (Mingeot-Leclercq and Tulkens, 1999), and protein kinase C (Kumar et al., 1997). The drug-lipid interactions can be involved in the actual mechanism of action or in the adverse effect of drugs, as proposed for the lung toxicity of amiodarone (Reasor and Kacew, 1996), cardiotoxicity of doxorubicin (Goormaghtigh et al., 1982), and nephrotoxicity of aminoglycosides, cephalosporins, and amphotericin B (Kaloyanides, 1994; Mingeot-Leclercq and Tulkens, 1999). Displacement of peripheral membrane proteins by competition with drugs for the liganding lipids is also possible, as demonstrated for cytochrome *c* (Jutila et al., 1998). Understanding of drug-lipid interactions is crucial in the design of liposomal drug carriers (Drummond et al., 1999). Finally, the lipid membrane could represent the actual target for the drug (Kinnunen, 1991), as shown for amphotericin B (Bolard, 1986), and antimicrobial peptides such as magainin, cecropin, and defensin (Bechinger, 1997; Matsuzaki, 1998; 1999; Oren and Shai, 1998; Shai, 1999; Sitaram and Nagaraj, 1999), for which no protein receptors have been identified. Also, the membrane interactions of general anesthetics are believed to be important in the mechanism of action (Cantor, 1997a and references therein). The large number of different lipid species in cells and the organ specific variations in membrane lipid compositions are likely to be selected by evolution for certain purposes. The thorough understanding of drug-lipid interactions is important in the light of the examples given above.

Clinical relevance

For efficient absorption from the gastrointestinal tract a compound has to cross the plasma membrane of enterocytes lining the gut lumen. The majority of the compounds crosses the plasma membrane by passive duffusion through the lipid bilayer (Kansy et al., 2001). The main determinant for absorption thus being drug-lipid interactions. After the drug has reached the circulation it will redistribute in the body. The distribution can be limited due to some blood-tissue barriers, such as the blood-brain barrier (Krämer et al., 2001). The blood-brain barrier is formed by

endothelial cells sealed by tight junctions in brain capillaries, and several proteins are involved in the formation of tight junctions (Krämer et al., 2001). The blood-brain barrier is also metabolically very active and contains large amount of transport proteins (Krämer et al., 2001). However, most of the compounds going through these barriers must cross the lipid bilayer of the endothelial cells (Krämer et al., 2001). Thus, drug-lipid interactions are playing crucial role in the distribution of compounds across blood-tissue barriers.

Also, if the drug target is intracellular, the permeation through the plasma membrane of the target cell is of outmost importance. As different cells can have different plasma membrane compositions and physicochemical properties, a thorough knowledge of drug-lipid interactions would help the development of optimal properties for drug candidates.

Aminoglycosides bind to the acidic phospholipids which accumulate into the brush border of renal cells during hypoperfusion or ischemia (Molitoris et al., 1993). Megalinin transports aminoglycosides actively into the cells where they accumulate into lysosomes (Mingeot-Leclercq and Tulkens, 1999). The accumulation of aminoglycosides into lysosomes changes the properties of lysosomal membranes leading to formation of myelin bodies and eventually to phospholipidosis (Mingeot-Leclercq and Tulkens, 1999). By administering compounds which inhibit binding of aminoglycosides to acidic phospholipids the nephrotoxicity of these important antibiotics can be decreased (Mingeot-Leclercq and Tulkens, 1999). Several drugs are known to induce accumulation of lipids into the cell, a toxic side-effect called as phospholipidosis (Halliwell, 1997). Phospholipidosis is related to the effect of the drugs on the membrane lipid synthesis, transport, and degradation (Lüllman et al., 1978). The pathological implications include corneal opacity, decreased glomerular filtration, acute renal failure, and pulmonary fibrosis (Lüllman et al., 1978). Importantly, phospholipidosis can be reverted by stopping the administration of the drug (Lüllman et al., 1978). The high affinity of Amphotericin B towards ergosterol makes this drug a very specific antifungal agent and it is clinically used to treat systemical fungal infections (Gallis et al., 1990). Binding of amphotericin B onto fungal membranes causes the formation of drug-lipid complexes which causes deterioration of the membrane structure. The state of aggregation of amphotericin B

(monomers vs. aggregates) is important for the efficacy-toxicity profile. Monomeric amphotericin B is active only towards ergosterol containing fungal membranes whereas aggregated amphotericin B causes lysis in both ergosterol (fungal) and cholesterol (animal) containing membranes (Barwicz and Tancrède, 1997; Andreoli, 1974; de Kruijff and Demel, 1974; Cohen, 1992).

The toxicity of several pharmacological agents such as amphotericin B, doxorubicin, and paclitaxel can be reduced by encapsulating these compounds into liposomes (Adler-Moore and Proffitt, 1993; Janoff et al., 1993; Sharma and Straubinger, 1994). Additionally, the efficiency of these drugs can also be increased by their lipsomal encapsulation, as shown for doxorubicin (Drummond et al., 1999). As these drugs are incorporated into liposomes the life-time of the drugs in the body increases (Drummond et al., 1999). The blood vessels in the tumor tissue are more leaky than in normal tissue. Thus, the liposomes can enter into extravascular space of tumor tissue, but in normal tissue the entrance is limited and increases the drug concentration in the tumor tissue as compared to normal tissue (Drummond et al., 1999). Liposomal formulations of several drugs are already in clinical use.

1.8. DOXORUBICIN

Doxorubicin is used in the treatment of various cancers and it belongs to group of antineoplastic drugs called anthracyclines. **(Fig. 4)** The mechanism of action of doxorubicin seems to be associated with the binding of doxorubicin to double stranded DNA (Dollery, 1999a). However, the coupling of doxorubicin onto large macromolecules prevents the accumulation of the drug in cells and its access to DNA, but the cytotoxic effect of the drug is not lost (Tritton and Yee, 1982; Wingard et al., 1985; Barabas et al., 1991; Faulk et al., 1991). Another anthracycline, daunorubicin, activates sphingomyelinase in cell cultures leading to the degradation of sphingomyelin to ceramide and phosphocholine. The accumulation of ceramide induces apoptosis in these cells already at drug concentrations obtained *in vivo* (Jaffrézou et al., 1996). In this respect the activation of phospholipase A_2 by doxorubicin *in vitro* could be an important factor in modulating the pharmacodynamic effects of this agent (Mustonen and Kinnunen, 1991). The most feared side-effect of doxorubicin is its dose-dependent cardiotoxicity. Doxorubicin efficiently inhibits

mitochondrial respiratory chain activities. The inhibition is due to the binding of doxorubicin to cardiolipin, blocking the binding of respiratory chain enzymes to this lipid (Huart et al., 1984; Nicolay and de Kruijff, 1987). The association constants of doxorubicin to cardiolipin and DNA are almost equal ($1.8*10^6$ M^{-1} vs. $3.6*10^6$ M^{-1}), indicating that cardiolipin can compete with DNA as a drug binding site (Goormaghtigh et al., 1980). In light of the above findings, the molecular level knowledge of doxorubicin-lipid interactions is important in order to define its mechanism(s) of action and adverse effects.

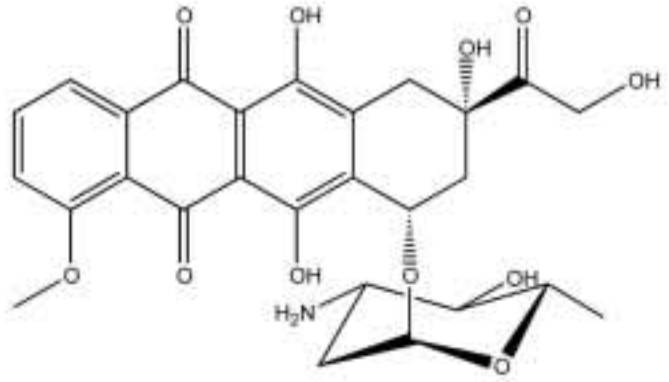


Figure 4. Chemical structure of doxorubicin.

Drug-lipid interactions of doxorubicin have been extensively investigated. Two types of membrane binding have been established for doxorubicin, one driven by electrostatic interactions and the other mainly by hydrophobic interactions (Duarte-Karim et al., 1976; Karczmar and Tritton, 1979; Burke and Tritton, 1985; Henry et al., 1985). In the latter binding mode the hydrophobic dihydroxyanthraquinone ring is intercalated between phospholipid acyl chains, whereas in the electrostatic type of binding the amino sugar of doxorubicin is interacting with the ionized phosphate of the acidic phospholipid (Karczmar and Tritton, 1979; Burke and Tritton, 1985; Henry et al., 1985; Fiallo and Garnier-Suillerot, 1986; Dupou-Cézanne et al., 1989). Phosphatidylethanolamine (PE) and acidic phospholipids enhance the binding of doxorubicin to membranes, possibly due to hydrogen bonding between PE and doxorubicin (Speelmans et al., 1997). This enhanced binding is of interest, as the acidic phospholipids are colocalized with PE into the inner leaflet of plasma membrane, suggesting a higher drug content on the inner leaflet as compared to outer

leaflet. The increase in membrane fluidity increases the accumulation of doxorubicin into cells (Burns and North, 1986), which is in agreement with the higher membrane partition of doxorubicin into fluid than gel phase membranes (Karczmar and Tritton, 1979).

Doxorubicin has very small effects on the thermal phase behaviour of zwitterionic phospholipids (Constantinides et al., 1986). However, when small amounts of acidic phospholipids are present, doxorubicin causes phase separation (Constantinides et al., 1986) and decreases T_m, the magnitude of the effect being dependent on the type of the acidic phospholipids (Tritton et al., 1978; Constantinides et al., 1986).

1.9. CYCLOSPORIN A

Cyclosporin A is an immunosuppressing drug used in the organ transplantation to prevent the graft rejection by the host immune system (Dollery, 1999b). **(Fig. 5)** CsA is composed of eleven amino acids, six of which are methylated, and one amino acid is D-isomer. The affinity of CsA towards intra-cellular protein cyclophilin is well established, and the inhibition of the function of cyclophilin-calcineurin complex is central to the mechanism of action of CsA (Hemar and Dautry-Varsat, 1990; O´Donohue et al., 1995).

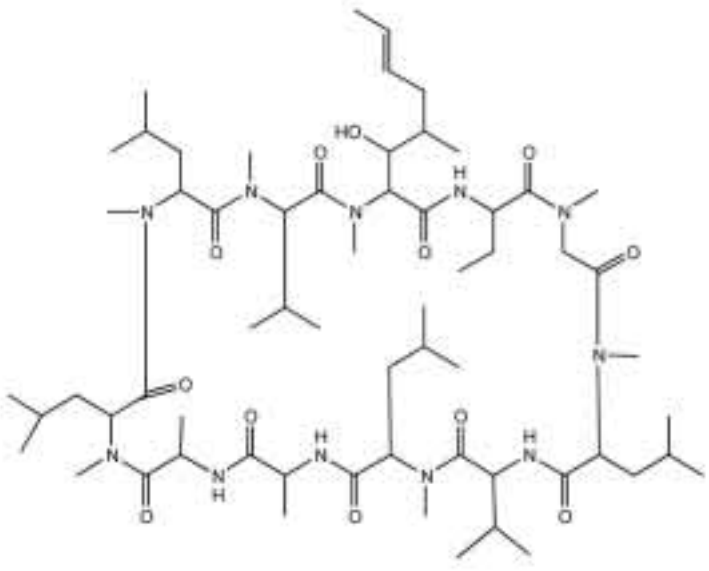


Figure 5. Chemical structure of cyclosporin A.

Due to its hydrophobicity, CsA readily partitions into lipid membranes. Membrane partitioning is a prequisite for the efficient interaction of compounds with the PgP (Romsicki and Sharom, 1999; Lu et al., 2001). CsA inhibits the function of the PgP which is involved in the excretion of bile salts from liver cells into bile duct. The impaired excretion of bile salts is involved in CsA induced cholestasis (Yasumiba et al., 2001). The cholestasis was accompained with concominant increase in cholesterol/phospholipid ratio and increased DPH anisotropy, i.e. decreased acyl chain movements (Yasumiba et al., 2001). Accordingly, the binding of CsA into lipid bilayers can be expected to cause changes in the physical properties of membranes as has been reported. CsA decreases the main transition enthalpy and temperature of vesicles composed of saturated DPPC, and abolishes the pretransition (O´Leary et al., 1986; Wiedmann et al., 1990). NMR measurements revealed CsA to increase acyl chain order in DPPC liposomes at temperatures above T_m. At temperatures below T_m of DPPC CsA induced disorder in the acyl chains (Wiedmann et al., 1990). These changes on acyl chain order below and above T_m of DPPC are similar to those caused by cholesterol (Wiedmann et al., 1990). The effect of CsA on membranes in living cells was quite opposite, as at 37 ^{o}C CsA decreased the fluorescence polarization of DPH, indicating decreased acyl chain order (Drori et al., 1995). As these results are compared to CsA induced changes in canalicular membrane lipid dynamics (Yasumiba et al., 2001), it is evident that CsA induces cell specific changes in membrane lipid dynamics. Myelin basic protein induced fusion of lipid membranes was inhibited by CsA, and the fluid-H_{II} phase transition temperature of dielaidoylphosphoethanolamine was increased at low CsA-to-lipid ratios and decreased at high contents of CsA (Epand et al., 1987).

1.10. CLOZAPINE, CHLORPROMAZINE, AND HALOPERIDOL

Neuroleptic drugs are used in the treatment of schizophrenia and other types of psychiatric disorders. **(Fig. 6)** These drugs have been shown to affect the functions of various receptors, such as adrenergic, muscarinic, histamine, 5-hydroxytryptamine, and dopamine receptor families (Bymaster et al., 1999). Studies on other than neurotransmitter receptor mediated effects are limited for CLZ and HPD, whereas CPZ is a very extensively investigated neuroleptic drug. CPZ has been reported e.g. to

inhibit protein kinase C (Singh et al., 1992), and to alter the activities of enzymes of lipid metabolism (Hoshi and Fujino, 1992; Heiczman and Tóth, 1995). The observed increase in the cellular content of acidic phospholipid and increased unsaturated/saturated lipid ratio induced by CPZ could represent adaptive responses (Stuhne-Sekalec et al., 1987).

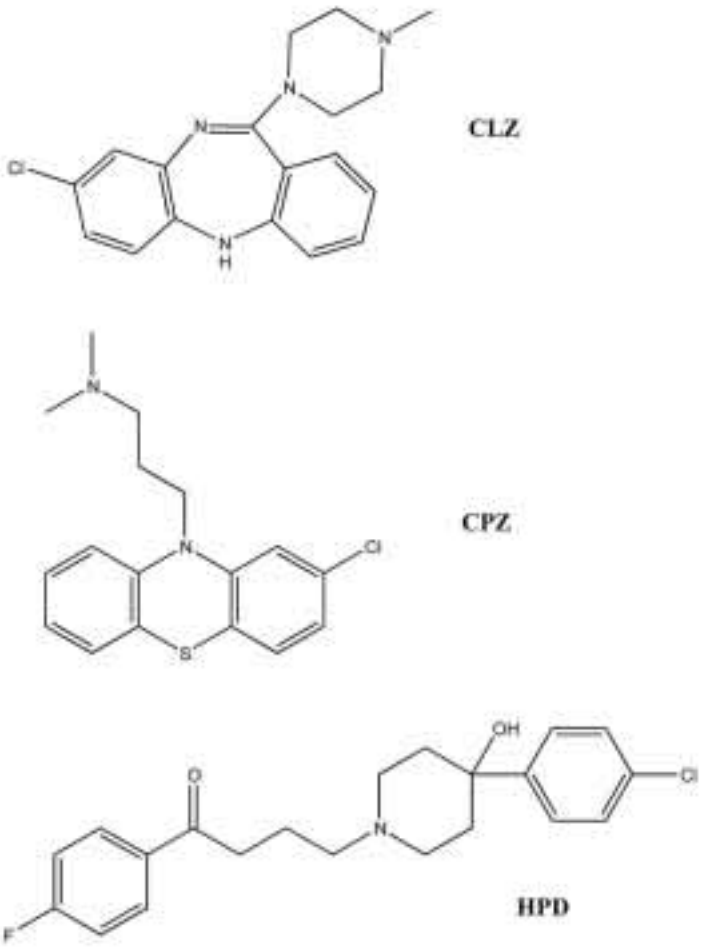


Figure 6. Chemical structures of clozapine (CLZ), chlorpromazine (CPZ), and haloperidol (HPD).

CPZ binds to the headgroup region of acidic phospholipid, forming a 1:1 complex (Kubo and Hostetler, 1985; Stuhne-Sekalec et al., 1987). It also penetrates into the acyl chain region of phospholipid membranes (Römer and Bickel, 1979; Kubo and Hostetler, 1985). Depending on membrane lipid composition and phase state CPZ has been reported to cause both an increase and decrease in the acyl chain order in membranes (Neal et al., 1976; Römer and Bickel, 1979; Zubenko and Cohen, 1985).

In gel phase phospholipid membranes CPZ induces the formation of fluid domains (Hanpft & Mohr, 1985). Binding of HPD to phospholipid membranes increases disorder more in the interfacial region than in the hydrophobic core of the membrane (Palmeira & Oliveira, 1992). CPZ and CLZ are good antioxidants and decrease membrane lipid peroxidation (Dalla Libera et al., 1998) whereas HPD has been reported to have an opposite effect (Sawas & Gilbert, 1985). These effects might be of importance as lipid peroxidation has been shown to affect the affinity or number of binding sites in membranes for 5-hydroxytryptamine, muscarinic, α-adrenergic, and dopamine receptor ligands (Rego and Oliviera, 1995).

1.11. MODEL MEMBRANES

Why to use model membranes?

As described earlier in this chapter, the biomembranes contain large number of different lipids and proteins. The experiments on lipid-protein and drug-lipid interactions would initially be very complex to analyze, due to the multiple variables. By using well-defined model systems, the underlying physicochemical principles and specific interactions are more precisely interpreted. The dependence of PLA_2 activity on membrane lateral heterogeneity is well established in model membranes (Mouritsen and Biltonen, 1993; Burack et al., 1993; Burack and Biltonen, 1994; Hønger et al., 1996; Huang et al., 1998). Recently, the same membrane properties were shown to determine the PLA_2 activity also in biological membranes (erythrocytes), indicating that mechanisms obtained from simple model membranes also apply to complex biological systems (Harris et al., 2001).

Liposomes

Liposomes are spherical lipid vesicles formed by a single (unilamellar) or multiple (multilamellar) bilayers, enclosing small volumes of aqeous solution inside the vesicle (Bangham et al., 1965). The diameter of liposomes (∅ from ~30 nm to several μm) depends on the preparation technique. The choice of lipid compositions is limited, as not all lipids or lipid mixtures form planar membranes which are prequisite for liposome formation. The curvature of liposomes, and the lipid composition of a single liposome cannot be exactly controlled. Liposomes can be studied by various techniques, such as spectroscopy, X-ray diffraction, and differential scanning

calorimetry. Some of the techniques require the incorporation of probes, such as fluorescent dyes, spin-labelled molecules, and different isotopes. Liposomes with diameter of several μm can be visualized directly by phase contrast, fluorescence, and confocal microscopy. Bilayer curvature depends on the liposome diameter, with small unilamellar vesicles (∅<50 nm) having the highest curvature. For giant unilamellar liposomes (∅>1 μm) the average bilayer curvature is practically the same as for planar membranes when considering curvature at molecular level. For a liposome composed of one lipid species, the bilayer packing defects are highest for small vesicles. This property (curvature) of liposomes should be taken into account while comparing the results obtained from measurements using liposomes of different size.

Monolayers

Monomolecular films on the surface of an aqueous solutions are called (Langmuir) monolayers. At air-water interfaces amphiphilic molecules are oriented so that hydrophobic parts are exposed to the air and the hydrophilic regions towards the aqueous phase. Monolayers form an excellent model for studying ordering in two-dimensions, with two thermodynamical variables, temperature and pressure, being readily controlled (Kaganer et al., 1999; Brockmann, 1999). In addition, the lipid composition and lateral packing can be controlled independently from membrane curvature (Kaganer et al., 1999; Brockmann, 1999). The classical way to characterize monolayers is to measure surface pressure-area isotherms. In addition, the properties of monolayers can also be characterized by other techniques, such as surface potential measurements, and different imaging techniques, the latter providing information on the lateral organization of the monolayer (Möhwald, 1995). Lipid monolayers exhibit phase transitions similar to lipid bilayers. The liquid-expanded (LE) phase is somewhat similar to the fluid phase in lipid bilayers, and a liquid-condensed (LC) phase somewhat corresponds to a gel phase. Acyl chain mobility is high in the LE phase, intermolecular interactions low, and lateral diffusion is $>10^{-8}$ cm^2/s (Möhwald, 1995). In contrast, LC phase is characterised by highly ordered acyl chains (oriented perpendicular to the surface), strong intermolecular interactions, and lateral diffusion $<10^{-10}$ cm^2/s (Möhwald, 1995). The phase coexistence can be visualized by fluorescence microscopy. The contrast in the images is due to different dye solubility or quantum yield in different phases (Möhwald, 1995). Fluorescence microscopy of

lipid monolayers have been used to study lateral organization of lipid monolayers (Nag et al., 1991; Maloney and Grainger, 1993). Fluoresecence microscopy of lipid monolayers has been used to demonstrate phospholipase A_2 and cholesterol oxidase induced changes in membrane heterogeneity (Grainger et al., 1989; 1990; Slotte, 1995), phase coexistence (Weis, 1991), and domain formation induced by Ca^{2+} (Eklund et al., 1988) and by electric fields (Lee et al., 1994). In giant unilamellar vesicles (∅> 1 μm) lipid domains of similar size and morphology as in lipid monolayers have been reported (Bagatolli and Gratton, 2000).

2. AIMS OF THE PRESENT STUDY

i) to study the effects of membrane properties on the drug-lipid interactions of model drugs: chlorpromazine, clozapine, cyclosporin A, doxorubicin, and haloperidol. The membrane properties of interest were lateral packing and lateral heterogeneity. Also, the effect of cholesterol and acidic lipids were studied.

ii) to study the effect of chlorpromazine, clozapine, cyclosporin A, and haloperidol on membrane lateral organization.

iii) to compare the effects of surface tension and osmotic pressure on the interfacial dynamics of phospholipid bilayers.

3. MATERIALS AND METHODS

3.1. MATERIALS

Except for cyclosporin A (Novartis, Basel, Switzerland) all compounds were obtained from commercial suppliers. Deionized water used for buffers was Millipore filtered (Millipore, Bedford, MA, USA). Concentrations of non-fluorescent lipid and drug stock solutions were determined gravimetrically using a high precision electrobalance (Cahn Instruments, Inc., Cerritos, CA, USA). For fluorescent compounds the concentration were determined spectrophotometrically.

3.2. PREPARATION OF LIPOSOMES

Lipids were mixed in desired molar ratio in chloroform. The mixtures were dried under a stream of nitrogen, and the dry residues were kept under reduced pressure for at least 2 h to remove the last trace of solvent. Samples were hydrated in a buffer for 30 min at a temperature of approx. 10 ^{o}C above main transition temperature to yield multilamellar liposomes (MLVs). For DSC measurements the MLVs were maintained on ice overnight before measurement. When using large unilamellar vesicles (LUVs) the MLV dispersions were subsequently extruded (Macdonald et al., 1991) through polycarbonate filter (pore size 0.1 μm, Millipore, Bedford, MA, USA) using Liposofast-Pneumatic (Avestin, Ottawa, Canada) (Macdonald et al., 1991). For osmotic pressure studies (IV) the MLVs were freeze-thawed five times before extrusion.

3.3. DIFFERENTIAL SCANNING CALORIMETRY (DSC) (I-III)

Heat capacity scans of liposome samples (total lipid concentration 0.4 mM (III) or 0.7 mM (I-II)) were recorded with a high-sensitivity differential scanning microcalorimeter (VP-DSC, Microcal Inc., Northampton, MA, USA), operated at a heating rate of 0.5 ^{o}C/min. The data were analyzed using commercial software (Origin, Microcal Inc., Northampton, MA, USA). Transition enthalpies are expressed as kilojoules per mole of phospholipid and were determined by integration of the

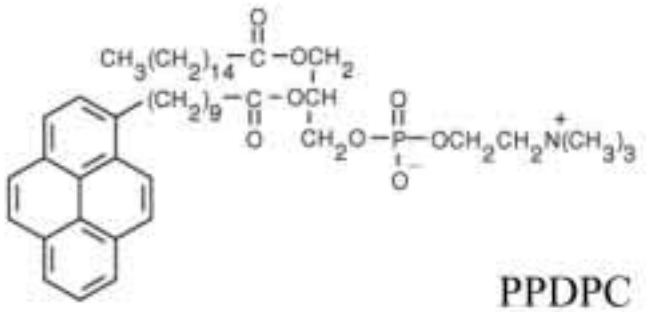
peaks, using the internal calibration of the instrument as a reference. The deviation from the baseline was taken as the beginning of the transition and the point of return to the baseline as the end of the transition. The pretransition temperature (T_p) was determined as the peak of the smaller endotherm, and the maximum in excess heat capacity was taken as the lipid main transition temperature (T_m). Data points illustrated represent the mean for two or three separate samples, and the error bars indicate standard deviation.

3.4. RESONANCE ENERGY TRANSFER (I)

Distance between two molecules can be determined by using resonance energy transfer. The efficiency of the transfer depends on the average distance between the donor and the acceptor being inversely proprotional to r^6 (Stryer, 1978). Electronic energy is efficiently transferred from a fluorescent energy donor to a suitable acceptor up to a distances of 60 Å (Stryer, 1978). Additionally, orientation of the donor emission and acceptor absorption dipoles, and the magnitude of the spectral overlap of the donor emission and acceptor emission spectra determine the efficiency of the energy transfer process (Stryer, 1978). Doxorubicin has a broad absorption peak centered at 480 nm, which overlaps the emission spectrum of pyrene (Mustonen and Kinnunen, 1991). With excitation of the sample at 344 nm and measuring pyrene (donor) emission at ~480 nm it is possible to measure the binding of doxorubicin (acceptor) to lipid membranes containing pyrene (Mustonen and Kinnunen, 1991, 1993; Kôiv and Kinnunen, 1994). The membrane association of doxorubicin is evident as a decrease in pyrene emission intensity. Although the decrease in the fluorescence intensity correlates with the number of doxorubicin molecules bound to the liposomes the determination of the actual number of drug molecules bound to the vesicles is not possible, because at low surface occupancy the number of donors quenched by each bound acceptor is large. However, upon increasing surface occupancy by doxorubicin the decrement in emission intensity vs. the number of additional drug molecules bound diminishes in a progressive manner. Quantitation of resonance energy transfer processes of this type has been shown to be complex (Drake et al., 1991).

3.5 PYRENE FLUORESCENCE EMISSION (I)

Steady-state fluorescence measurements were carried out with a Perkin-Elmer LS50B spectrofluorometer equipped with magnetically stirred, thermostated cuvette compartment. The data were analyzed using the dedicated software provided by Perkin-Elmer. Membrane lateral organization was monitored using pyrene labelled phospholipid analogs, PPDPC and PPDPG. **(Fig. 7)**



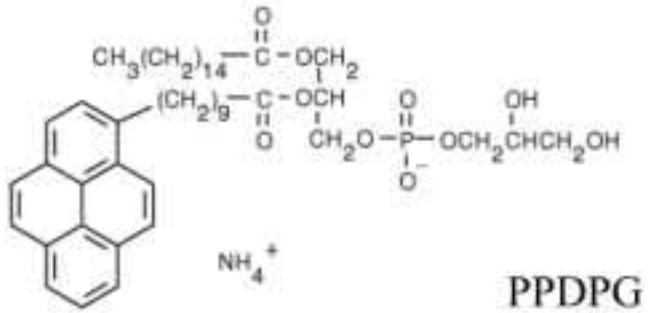


Figure 7. Chemical structures of PPDPC and PPDPG.

The final lipid concentration in steady-state measurements was 25 μM. Monomeric pyrene relaxes to the ground state by emitting photons at ~378 nm or at ~400 nm, the peak intensity and maximum emission wavelength depending on the solvent polarity (Kinnunen et al., 1993; Duportail and Lianos, 1996). During its lifetime, excited-state pyrene can form a characteristic short-lived complex, excimer with a ground-state pyrene. This complex relaxes back to ground-state by emitting quanta as a broad and featureless band centered at ~ 480 nm (I_e). In the absence of possible quantum mechanical effects the excimer-to-monomer ratio (I_e/I_m) depends on the rate of collisions between pyrenes (Kinnunen et al., 1993). Consequently, for pyrene-

containing phospholipid analogs the value of I_e/I_m reflects the lateral diffusion as well as the local concentration of the fluorophore in the membrane (Kinnunen et al., 1993). Excitation was at 344 nm while pyrene monomer and excimer emission was recorded at ~ 378 nm and at ~ 480 nm, respectively. The samples were initially equilibrated at lowest T (~28 ^{o}C) for 10 minutes. After recording the spectra, T was increased. The sample was equilibrated for 3 minutes in the new T before measuring the spectra.

3.6. LAURDAN FLUORESCENCE MEASUREMENTS (IV)

Laurdan **(Fig. 8)** steady-state fluorescence measurements were carried out with a Perkin Elmer LS50B spectrofluorometer equipped with a magnetically stirred cuvette compartment thermostated with a circulating waterbath (Haake F6/Haake C25, Karlsruhe, Germany). The data were analyzed by dedicated software from Perkin-Elmer. The excitation wavelength was 350 nm and emission was monitored at 440 and 480 nm. The emission generalized polarization (GP) was calculated using equation:

$$GP = (I_{440}-I_{480}) / (I_{440}+I_{480})$$

where I_{440} and I_{480} are the emission intensities measured at 440 and 480 nm (Parasassi et al., 1998). Importantly, these measurements do not relate to emission polarization but to polarity of the fluorophore surroundings. Final lipid concentration was 25 μM. Measurements were done at 25 ^{o}C.

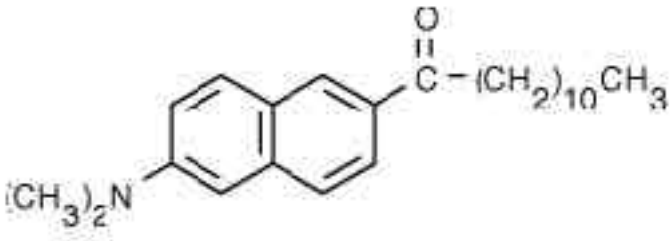


Figure 8. Chemical structure of Laurdan.

3.7. STOPPED-FLOW MEASUREMENTS (I)

The kinetics of binding of doxorubicin to liposomes was measured using a stopped-flow spectrofluorometer (Olis RSM 1000F, On-Line Instruments, Inc., Bogart, GA, USA). A computer controlled circulating waterbath (Neslab Instruments, Portsmouth, NH, USA) regulated the temperature in the reaction chamber and in the syringes. The final concentrations of phospholipid and doxorubicin were 25 and 4 μM. Excitation wavelength was at 344 nm, and fluorescence emission was recorded between 375 and 525 nm. The fluorescence decays for each sample were monitored using scanning rates of 1000, 62, and 10 scans/s with the respective collection times of 0.25, 5, and 96 s.

Values for the given halftimes of the reactions represents averages from at least three separate measurements. Data with two fluorescence decay processes were fitted by the equation

$$Y=A_1 \times e^{-k1.t} + A_2 \times e^{-k2.t}$$

which was solved with nonlinear least-squares fitting procedures by both Levenberg-Marquardt algorithm and Successive Integration method using the routines of the software provided by the instrument manufacturer. The best-fit parameters A_1 and A_2 represent the amplitudes of the processes and k1 and k2 the respective kinetic rate constants. In 96 seconds measurements the photobleaching of pyrene due to the 450 W xenon lamp irradiation became evident as an additional slow process. As this bleaching was very slow ($t_{1/2} \approx$ 115 s) it did not interfere with the recording of the fluorescence intensity decays due to the membrane association of doxorubicin. However, because of the large difference in the halftimes of the different components the routines of the software did not allow to obtain accurate values for the amplitudes for the two exponential processes measured. Yet, the relative amplitude (RA) of the fast fluorescence decay seen within the 250 ms timedomain can be calculated from the data by equation:

$$RA=\Delta I/I_0$$

where I_0 is the initial level of fluorescence of the LUVs prior to the rapid quenching by doxorubicin and ΔI is the amplitude of the rapid fluorescence decay (decrement in I). **(Fig. 9)**

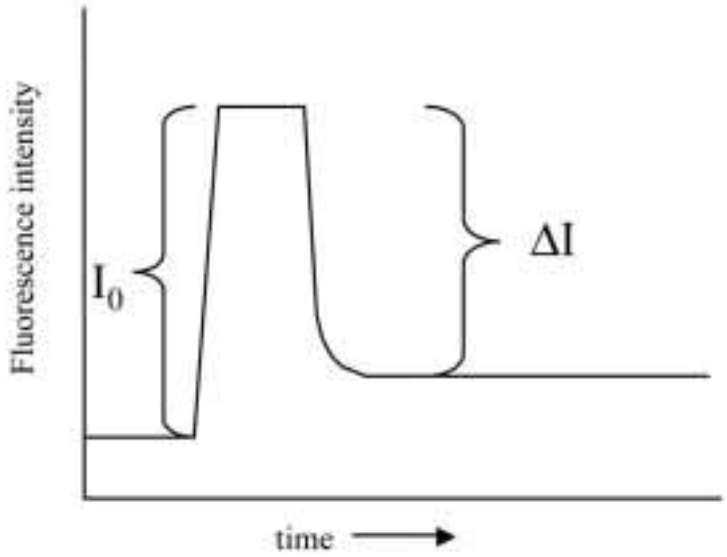


Figure 9. Relative amplitude (RA) of the rapid fluorescence quenching was calculated by equation $RA=\Delta I/I_0$. The increase in fluorescence (I_0) is due to the addition of pyrene labelled liposomes.

3.8. MONOLAYER PENETRATION MEASUREMENTS (II-III)

Penetration of drugs into monomolecular lipid films was measured using magnetically stirred circular wells. Surface pressure (π) was monitored with a platinum Wilhelmy plate (II) or wire probe (III) attached to a microbalance (KSV2200, KSV instruments, Helsinki, Finland (II), and μTrough S, Kibron, Inc., Helsinki, Finland (III)). The aqueous phase was 5 mM Hepes, 0.1 mM EDTA (pH 7.4). Lipids were spread on the air-buffer interface in chloroform (approx. 1 mg/ml) and were allowed to equilibrate for 20 min so as to reach different initial surface pressures (π_0) before the addition of drug (in DMSO) into the subphase. The increment in π after the addition of drug was complete in 2-20 minutes depending on the drug and lipid composition. $\Delta\pi$ was take to be the difference between the initial surface pressure (π_0) and the value observed after the intercalation of the drug into the film. The data are represented as $\Delta\pi$ vs. π_0, thus revealing the decrement in the intercalation of drug into lipid monolayer upon increasing lateral packing density of the film. Final concentration of DMSO was <1 vol-%, and at this concentration DMSO had no measurable effect as such on the π. All monolayer measurements were done at ambient temperature (~22 ^{o}C).

3.9. COMPRESSION ISOTHERMS (III)

Compression isotherms were recorded using 111.1 cm^2 (width 55 mm, length 202 mm, subphase volume 22 ml) trough. Surface pressure (π) was monitored with a metal alloy probe hanging from a high precision microbalance (μTrough S, Kibron Inc.). For compression isotherms and fluorescence microscopy the indicated mixtures of lipids and neuroleptic drugs were dissolved in a mixture of hexane/isopropanol/water (70/30/2.5, by vol.). These solutions were spread on the air-buffer (20 mM Hepes, 0.1 mM EDTA, pH 7.0) interface. After 5 min equilibration the film compression was started using two symmetrically moving barriers. The compression rate was in all measurements one $Å^2$/acyl chain/min. Data is represented as π vs. area/acyl chain, where each lipid molecule consists of two acyl chains. In the calculations one drug molecule is taken as equivalent to one acyl chain. All monolayer measurements were done at ambient temperature (~22 ^oC). The mean molecular areas occupied by the drugs (equivalent to one acyl chain) in the film at any given surface pressure were calculated using the following equation:

$$A_D = (A_T - A_L) / X_D$$

where A_T is the mean molecular area of the molecules in the presence of the indicated drug, A_L is the surface area of the lipids in the absence of the drug, A_D is the surface area of the drug, and X_D its mole fraction in the film. The equation applies to the situation with either ideal mixing or complete immiscibility, i.e. not involving molecular interactions, condensing effects, or non-ideal partitioning of the drugs. However, the results allow the qualitative comparison of the drugs.

3.10. FLUORESCENCE MICROSCOPY (II, III)

For fluorescence microscopy a Langmuir trough equipped with a quartz window on the bottom was placed on the stage of a Zeiss IM-35 inverted fluorescence microscope. Compression isotherms were recorded as described above with slight modifications, as follows. After the desired target pressure was reached by continuous compression by two barriers the film was allowed to stabilize for 10 min before the image was recorded through a Nikon ELWD (20x) objective. NBD-PC (X=0.02) was

used as a fluorescent tracer for microscopy. The excitation and emission wavelengths were selected with filters transmitting in the range 420-480 nm and > 500 nm, respectively. Fluorescence images were viewed with a Peltier-cooled digital camera (Hamamatsu C4742-95, Hamamatsu, Japan) connected to a computer. During the 10 min equilibration time a small decrease in π was observed, reflecting the relaxation of the monolayer. It is to be emphasized that the images obtained are unlikely to represent true equilibrium. The solid domain growth kinetics has an effect on the shape and size of these domains (Miller and Möhwald, 1987), thus the relaxation of the monolayer is a potential source of error in the measurements. Yet, the results should be amenable to comparison as the equilibration times and compression rates were kept identical. Moreover, the observed domain morphologies were reproducible. All measurements were performed at ambient temperature (~22 ^{o}C).

3.11. OSMOLARITY AND SURFACE TENSION MEASUREMENTS (IV)

The freezing point depression method (Micro-Osmometer Model 3300, Advanced Instruments Inc., Norwood, MA, USA) was used to obtain Π vs. osmolality curve for choline chloride. All measurements were done in duplicate. The difference between these duplicate assays was 0-16 mosm/kg, with an average difference of 6 mosm/kg. For betaine and sucrose the Π vs. osmolality data were retrieved from the homepage of the Laboratory of Physical & Structural Biology (http://dir.nichd.nih.gov/Lpsb/docs/OsmoticStress.html). The measurements were done by the vapor pressure method.

The effect of betaine, choline chloride, and sucrose on the surface tension of water was measured by a multichannel microtensiometer (MultiPi WS2, Kibron Inc., Helsinki, Finland). Sample volume was 50 µl per well. The instrument uses the du Nouy technique with the wire probes attached to the microbalance sensor heads. All measurements were done at ambient temperature (~22^{o}C) and for each solute concentration at least six individual samples were measured. The surface tension recorded for pure water was 72.8±0.1 mN/m, in keeping with the literature (Adamson, 1990).

4. RESULTS

4.1. MEMBRANE INTERACTIONS OF DOXORUBICIN (I)

Kinetics of binding of doxorubicin to liposomes

Kinetics of binding of doxorubicin to liposomes has not been reported. Therefore it was of interest to study whether the two different binding modes, electrostatic and hydrophobic, could be detected by the association kinetics of doxorubicin into the liposomes. Additionally, the effect of dynamic lateral heterogeneity on the kinetics of binding was investigated. The resonance energy transfer from pyrene to doxorubicin was used to assess binding of this drug to phospholipid LUVs. PPDPC (X=0.03) was incorporated into neutral POPC liposomes. POPC was gradually substituted by POPG in order to obtain negative charges into the liposomes. These lipids have been shown to mix ideally in absence of divalent cations (Findlay and Barton, 1978; Garidel et al., 1997). At X_{PG}>0.03 PPDPG was used as the resonance energy donor, and at X_{PG}=0.01 and X_{PG}=0.02 PPDPC was gradually substituted by PPDPG.

Effect of PG content

The binding of doxorubicin to fluid PC liposomes was observed to have a half-time ($t_{1/2}$) of approx. 2 s. **(Fig. 10)** At X_{PG}<0.04 only one fluorescence decay was observed, with $t_{1/2}$ remaining at ~2 s. At X_{PG}=0.04 the decay of fluorescence became two-exponential, the second fluorescence decay having $t_{1/2}$ ~1 ms. The fluorescence decay remained two-exponential upto X_{PG}=1.00. After the appereance of the second, fast fluorescence decay, the $t_{1/2}$ of the slower decay continuosly increased, whereas the $t_{1/2}$ of the faster decay remained within 1-4 ms. The relative amplitude (RA) of the fast fluorescence decay reached saturation at approx. X_{PG}=0.25. At low PG content $t_{1/2}$ of the fast fluorescence decay varied in an irregular manner, reaching a maximum at X_{PG}=0.065, and at X_{PG}=0.10, while a minimum in $t_{1/2}$ was evident at X_{PG}=0.09.

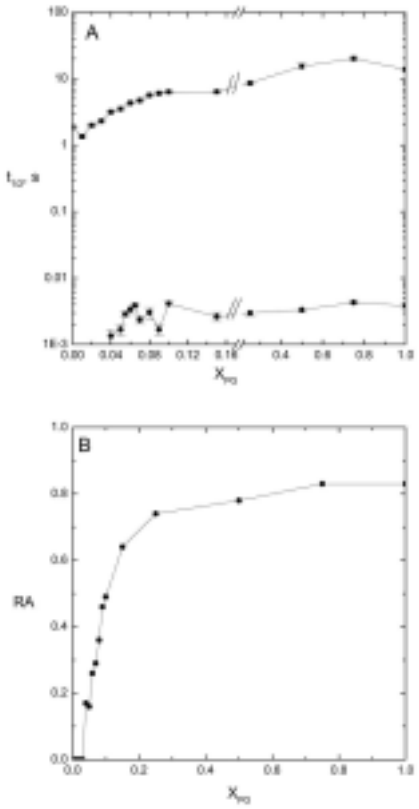


Figure 10. *Panel A)* Kinetics of binding of doxorubicin to liposomes as a function of PG content. *Panel B)* Relative amplitude (RA) of the fast fluorescence quenching (●) as a function of X_{PG}.

The increment in $t_{1/2}$ of the slower fluorescence decay is likely to result from decreased drug concentration in the buffer after the rapid membrane association. This was supported by the finding that the kinetics of binding of doxorubicin onto zwitterionic PC liposomes is dependent on the doxorubicin concentration (*Söderlund et al., unpublished observation*).

Doxorubicin has been shown to penetrate through the lipid bilayers into the liposomes, but the translocation of doxorubicin is rather slow, requiring 5 to 10 minutes to reach the equilibrium (Praet et al., 1993). Accordingly, it is unlikely that this process would contribute to the fluorescence changes reported here.

Screening of electrostatic charges on PC/PG (75:25) liposomes by NaCl and $CaCl_2$

The appereance of fast fluorescence decay in the presence of PG suggested this process to involve electrostatic interactions. In order to verify electrostatic interactions in the binding the effect of NaCl and $CaCl_2$ were studied. NaCl completely abolished the fast fluorescence decay at [NaCl]>50 mM. Also, at

[$CaCl_2$]>150 μM the fast fluorescence decay was no longer observed. These findings indicate that fast fluorescence decay involved electrostatic interactions between doxorubicin and liposomes, and clearly indicate that the two binding modes can be separated by their respective half-times. **(Fig. 11)**

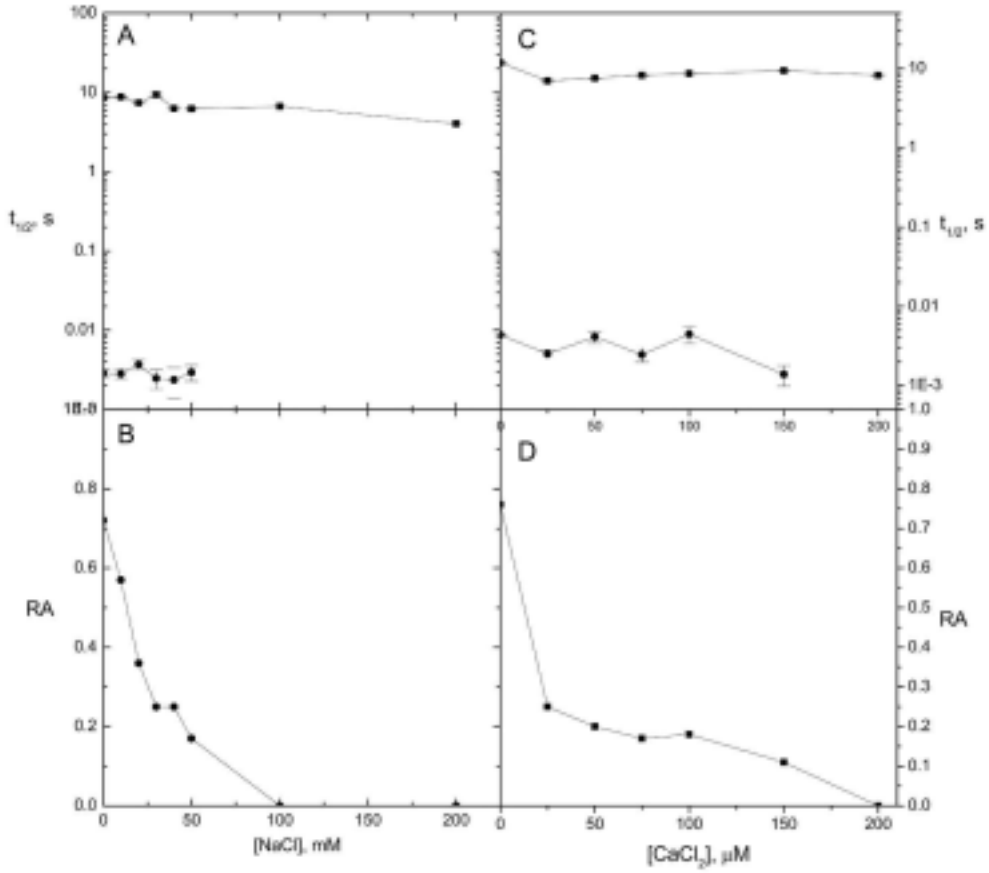


Figure 11. Effects of NaCl on the kinetics (*panel A*) and RA (*panel B*) of the binding of doxorubicin to PC/PG LUVs (X_{PG}=0.25). *Panel C)* illustrates the effects oc $CaCl_2$ on the kinetics and *panel D)* on the RA.

Effect of membrane lateral heterogeneity

The strong dependence of the fast electrostatic driven association of doxorubicin to X_{PG} makes it possible to use this process to probe the initial organization of the liposome surface before the binding of doxorubicin occurs. The kinetics of binding of doxorubicin to DPPC/PPDPG (97:3) LUVs were measured in the course of the main transition. The fraction of negatively charged PPDPG was lower than the amount required for rapid, electrostatic binding to fluid POPC/POPG vesicles. Only rapid fluorescence decay was evident at temperatures below 34 ^{o}C. The fast fluorescence

decay ($t_{1/2}$~2 ms) was observed up to 37 ^{o}C, while its relative amplitude decreased as the temperature was increased, approaching zero at ~38 ^{o}C. Increasing the temperature from 37 to 38.5 ^{o}C prolonged $t_{1/2}$ of the fast component from ~1 ms to 0.2 s. The slower fluorescence decay ($t_{1/2}$~20 s) was evident and constant at temperatures 34-36 ^{o}C. Increasing temperature from 36 to 39 ^{o}C resulted a decrease in $t_{1/2}$ of the slow fluorescence decay, and at temperatures above ~39 ^{o}C only one fluorescence decay was observed ($t_{1/2}$ from 0.3 to 0.2 s), $t_{1/2}$ decreasing as temperature was increased.

In order to verify the two-exponential fluorescence decay to arise from the presence of acidic phospholipid, the binding of doxorubicin to liposomes composed purely of PC (DPPC/PPDPC, 97:3) was measured. At the temperature range studied (30-46 ^{o}C) only one fluorescence decay was evident for the LUVs composed purely by zwitterionic lipids. As the temperature was increased $t_{1/2}$ became progressively faster. At 34 ^{o}C a sudden decrease in $t_{1/2}$ was observed, corresponding to the temperature of the appeareance of slow fluorescence decay in DPPC/PPDPC liposomes. **(Fig. 12)**

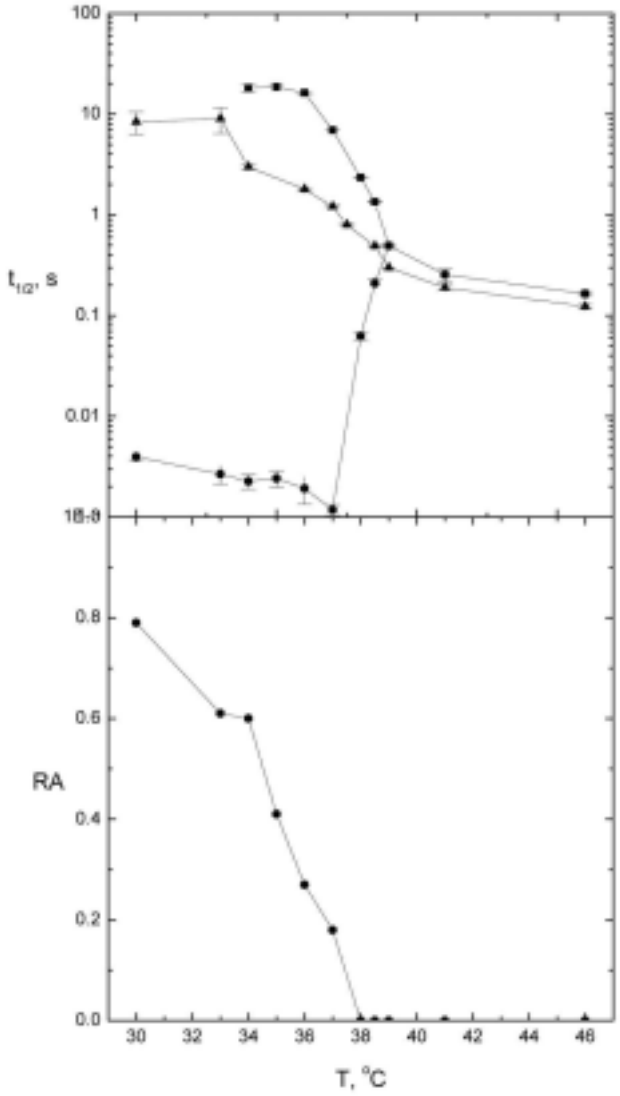


Figure 12. Dependence of doxorubicin binding kinetics and RA on temperature in DPPC LUVs.

Changes in the lateral distribution of PPDPC and PPDPG in the course of DPPC main transition

To obtain more information on the lateral organization during the main transition, the I_e/I_m of DPPC/PPDPC (97:3) and DPPC/PPDPG (97:3) LUVs was measured. PPDPC is efficiently excluded from gel phase matrix consisting of DPPC, leading to formation of domains enriched of the former lipid (Somerharju et al., 1985; Hresko et al., 1986; Marcie and Lentz, 1986). The similar mechanism could also result in the lateral segregation of PPDPG, but this has not been reported.

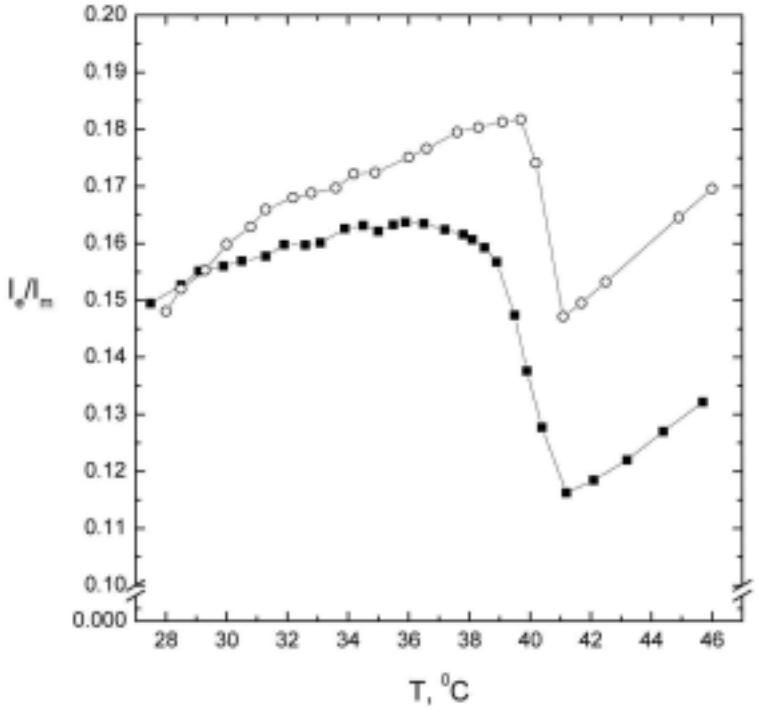


Figure 13. Temperature dependence of I_e/I_m values of PPDPC (X=0.03, O) and PPDPG (X=0.03, ■) in DPPC LUVs.

The values of I_e/I_m of PPDPG were increased with temperature up to ~34 ^{o}C whereafter a plateau was reached. At ~37 ^{o}C the I_e/I_m started to decrease in a progressive manner, until reaching a minima at ~41 ^{o}C. At temperatures above 41 ^{o}C I_e/I_m increased linearly in liquid crystalline state (fluid) liposomes. These findings suggest PPDPG molecules to be separated from the gel phase DPPC matrix. The segragation can be explained as follows. The bulky pyrene moiety in PPDPG perturbs the effective packing of gel phase DPPC. The segregation of PPDPG into domains

decreases the level of perturbation. The pyrene excimer formation of PPDPG was lower than that of PPDPC, likely reflecting electrostatic repulsions between PG headgroups of the former lipid derivative. **(Fig. 13)**

Differential scanning calorimetry was used to measure the effect of PPDPC and PPDPG (X=0.03) on the thermal phase behaviour of DPPC LUVs. The T_m of pure DPPC LUVs is 41.2 ^oC and it was decreased to 40.9 ^oC and 40.4 ^oC in the presence of PPDPC and PPDPG, respectively. **(Table I & Fig. 14)**

Table I. Compilation of the measured and deconvoluted DSC endotherms for the LUVs. The maximum in heat capacity in each curve is marked as T_m (experimental data) or T_{max} (in deconvoluted peaks). The deviation from the baseline is denoted as T_1 and return to the baseline as T_2. Numerical data for the main transitions are those illustrated in Fig. 14.

	DPPC	DPPC/PPDPC 97:3, mol/mol	DPPC/PPDPG 97:3, mol/mol
T_m	41.2 ^oC	40.9 ^oC	40.4 ^oC
T_1	37.7 ^oC	35.6 ^oC	34.0 ^oC
T_2	43.0 ^oC		
Peak 1			
T_{max}	40.5 ^oC	40.2 ^oC	38.9 ^oC
T_1	37.7 ^oC	36.2 ^oC	34.0 ^oC
T_2	43.4 ^oC	43.8 ^oC	43.5 ^oC
Peak 2			
T_{max}	41.2 ^oC	40.9 ^oC	40.5 ^oC
T_1	39.6 ^oC	39.8 ^oC	38.9 ^oC
T_2	42.8 ^oC	42.0 ^oC	42.0 ^oC
Peak P			
T_{max}		40.3 ^oC	40.0 ^oC
T_1		39.0 ^oC	36.9 ^oC
T_2		41.6 ^oC	43.3 ^oC

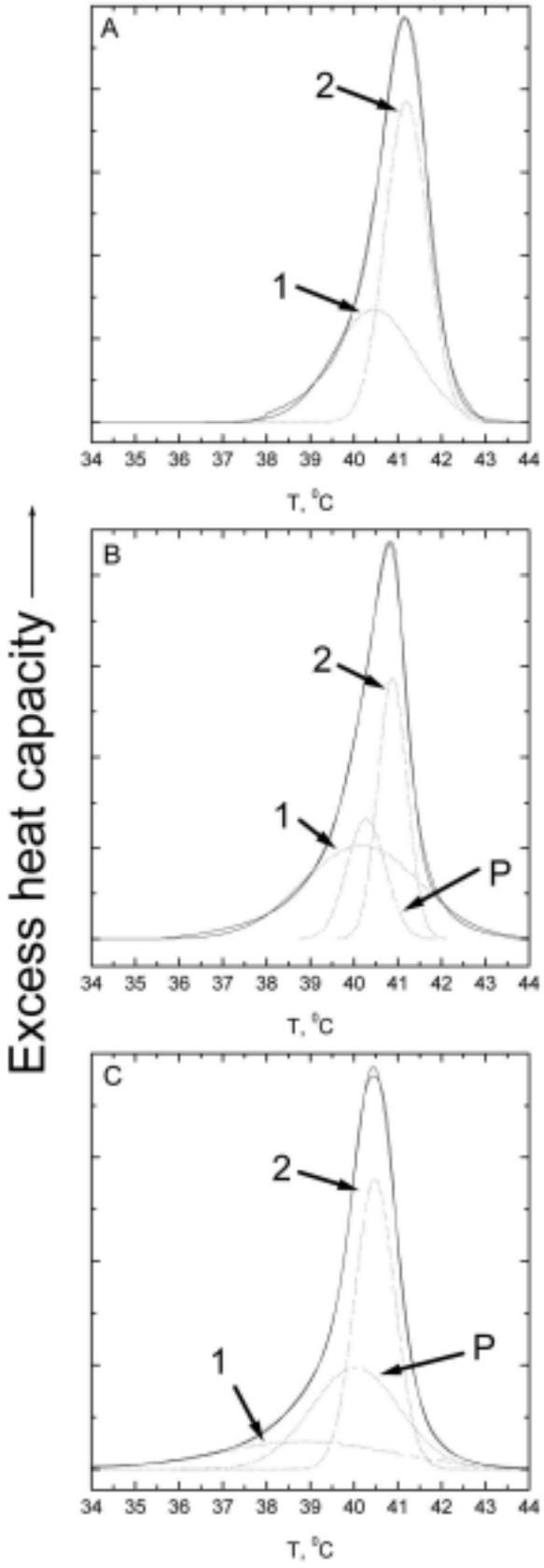


Figure 14. DSC endotherms of DPPC LUVs in absence (*panel A*) and presence of PPDPC (X=0.03, *panel B*) or PPDPG (X=0.03, *panel C*).

4.2. MEMBRANE INTERACTIONS OF CSA (II)

CsA has been demonstrated to induce cholesterol-like changes in acyl chain order. As cholesterol is abundant in the eucaryotic cell membranes it was of interest to investigate the interaction between CsA and cholesterol containing membranes.

Effect of CsA on the thermal phase behaviour of DMPC/cholesterol MLVs

The effect of CsA on the thermal behaviour of DPPC MLVs has been previously studied (O´Leary et al., 1986; Wiedmann et al., 1990). A low cholesterol content and a saturated phospholipid was selected for DSC measurements in order to obtain reliable endotherms. Therefore, the effects of CsA on the thermal behaviour of DMPC/cholesterol (10:1) MLVs were studied.

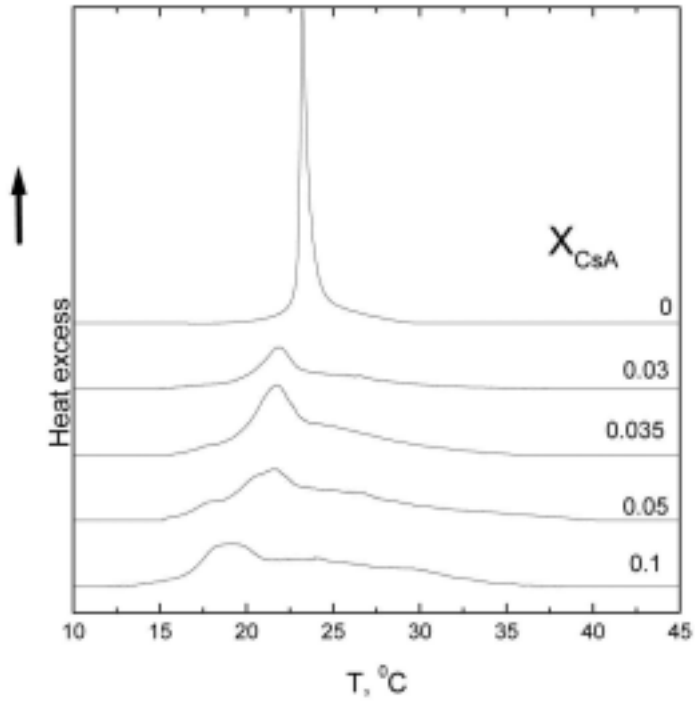


Figure 15. DSC endotherms of DMPC MLVs with increasing contents of CsA.

The effects of cholesterol on the phase behaviour of DMPC have been extensively studied, and cholesterol abolishes the pretransition already at low (X<0.05) contents, and broadens the main transition accompanied with decreased transition enthalpy (Koynova and Caffrey, 1998). A single endotherm was observed for DMPC/cholesterol MLVs with T_m~23.2 ^oC. CsA decreased the T_m of

DMPC/cholesterol MLVs in a concentration dependent manner, and at X_{CsA}=0.06 a drop (~0.6 ^{o}C) in T_m was observed.

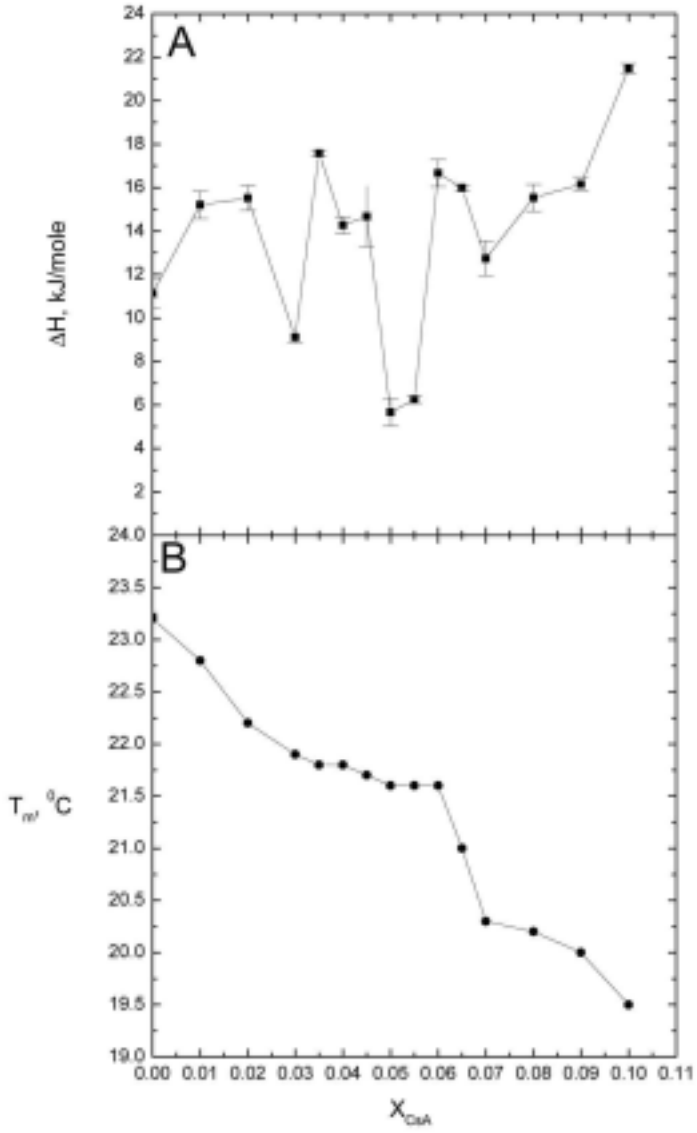


Figure 16. *Panel A)* Main transition enthalpy of DMPC MLVs as a function of CsA. *Panel B)* shows the effect of CsA on T_m of DMPC MLVs.

The presence of CsA caused a marked broadening of the endotherm, and multiple shoulders appeared into the endotherm, suggesting phase separations. The effect of CsA on the main transition enthalpy (ΔH) was very complex. At X_{CsA}=0.01 and 0.02 ΔH increased from ~11 kJ/mol (in absence of CsA) to ~15 kJ/mol. This increase was followed by a sudden drop at X_{CsA}=0.03, ΔH decreasing to ~9.1 kJ/mol. At

X_{CsA}=0.035 to 0.045 ΔH increased to ~14-18 kJ/mol, while at X_{CsA}=0.05 and 0.055 a sharp minimum in ΔH (~6 kJ/mol) was reached. As CsA content was further inceased ΔH remains at higher values than in absence of CsA. **(Figs. 15 & 16)**

Binding of CsA to lipid monolayers

The effect of lipid lateral packing and cholesterol on membrane association of CsA was studied. At surface pressures (π) below 19 mN/m the increase in π (Δπ) due to penetration of CsA into lipid monolayer was influenced by cholesterol. The slopes of the decrease of Δπ at π_0<19 mN/m extrapolated to zero at ~25 mN/m and ~24 mN/m for eggPC and eggPC/cholesterol (1:1), respectively. In eggPC monolayers the slope of Δπ changed abruptly at ~19 mN/m, and the new slope extrapolated to zero at ~35 mN/m. In the presence of cholesterol the change in the slope occurred at ~22 mN/m, and the new slope extrpolated to zero at ~31 mN/m. These results indicated that cholesterol decreased the penetration of CsA into lipid monolayers at high packing pressures (π_0> 19 mN/m). **(Fig. 17)**

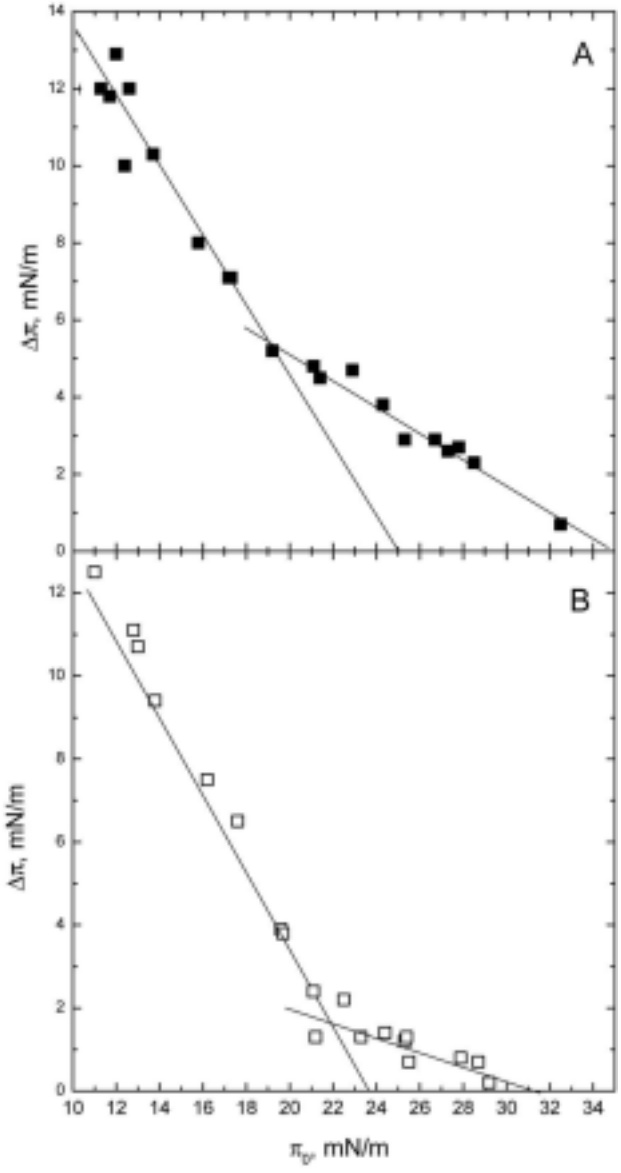


Figure 17. Increase in surface pressure (Δπ) of eggPC (*panel A*) and eggPC/cholesterol, (1:1, mol/mol, *panel B*) monolayers after addition of CsA (final drug concentration was 167 nM) into the subphase.

Influence of CsA on the lateral organization of lipid monolayers

The effects of CsA on the lipid domain morphology were visualized by fluorescence microscopy of lipid monolayers. NBD-PC was used as a fluorescent dye and it preferentially partitions into LE domains, and as a result the LC domains appear as dark, non-fluorescent areas (Weis and McConnell, 1985). Accordingly, fluorescence microscopy of phospholipid monolayers allows the visualization of the co-existence of LE and LC domains in the transition region (Weis, 1991). The calculated subphase concentration of CsA was 37.5 nM, i.e. assuming that all of the added CsA would dissolve into subphase.

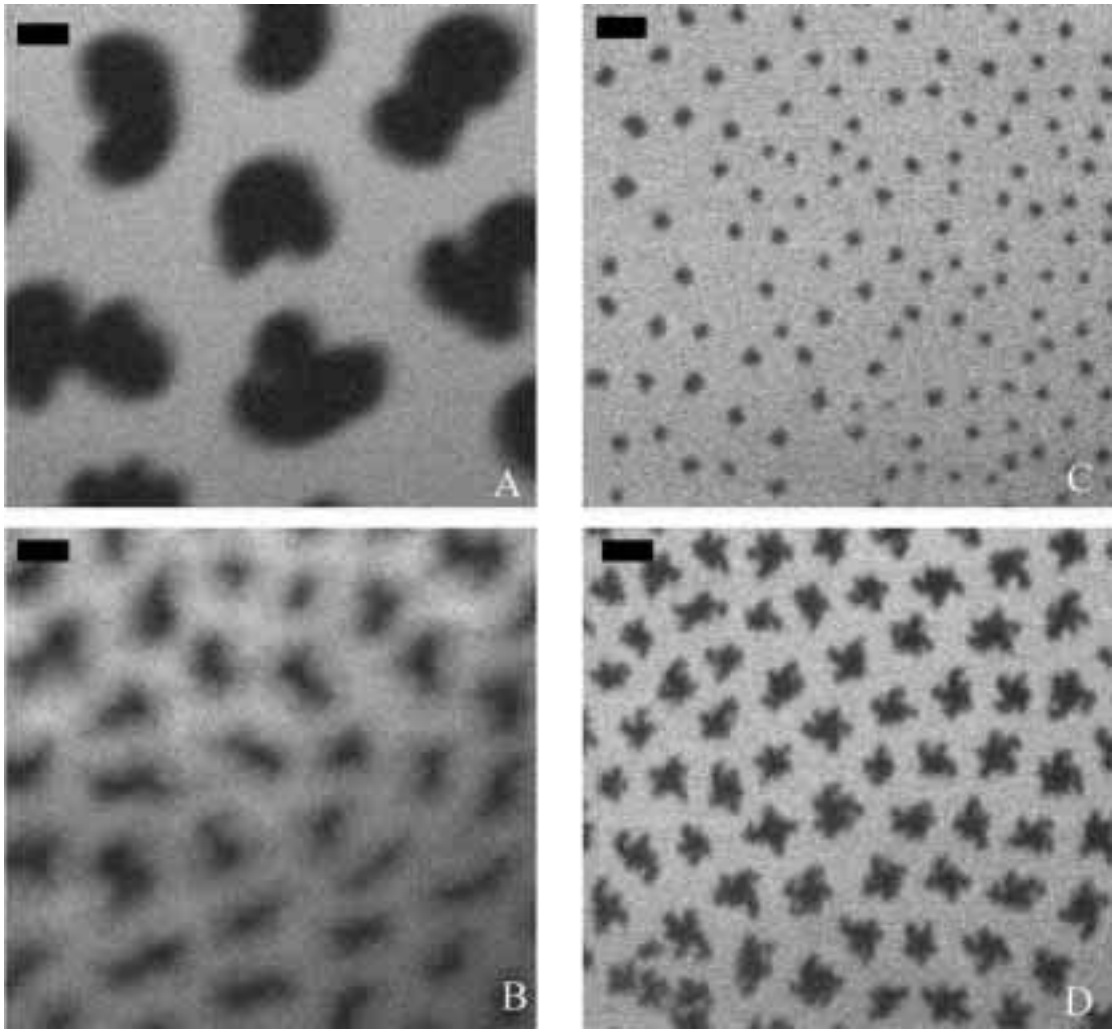


Figure 18. Fluorescence microscopy images of DPPC/NBD-PC monolayers in absence (*panels A and C*) and presence of CsA (*panels B and D*). The values of π were (*panel A*) 13.1 mN/m, (*panel B*) 14.1 mN/m, (*panel C*) 18.7 mN/m, and (*panel D*) 17.8 mN/m. NBD-PC (X=0.02) was incorporated as a fluorescent probe. Scale bar corresponds to 20 μm.

In the absence and presence of CsA the LC (dark) domains appeared at π~9-10 mN/m. In DPPC monolayers large solid domains were observed at π~13-14 whereas the presence of CsA caused a dramatic decrease in the size of these domains. In the presence of CsA, the relative interfacial boundary length of one domain was dramatically decreased as compared to domains in absence of CsA. In absence of CsA and at higher π values (15-25 mN/m) the fluid-solid domain boundaries become diffuse. In the presence of CsA the domain boundaries remained sharp even at higher π values (15-25 mN/m), indicating that CsA could stabilize the domain interfaces at higher π values. As π was increased from ~13 mN/m to 25 mN/m in the presence of CsA, the circular domains transformed into star-like structures with the area per domain increasing. **(Fig. 18)**

The presence of cholesterol (X=0.10) induced changes in the lateral organization of the monolayer. In the presence of cholesterol no large solid domains were observed, but instead a large reticular network of solid phase surrounded fluid domains. **(Fig. 19)** The observed domain morphology was in accordance with previously reported domain morphologies in DPPC/cholesterol (90:10) monolayers (Worthman et al., 1997). The domain morphology was in different from DPPC monolayers where solid domains were surrounded by fluid matrix. CsA completely destroyed the reticular network induced by cholesterol. In the presence of CsA in the DPPC/cholesterol monolayer only small circular solid domains were observed in fluid matrix. As π was increased the number of solid domains increased but their size remained practically the same. The increase in the number of LC domains per image as π was increased indicates a larger fraction of the monolayer to be in LC phase. During the monolayer compression, the mean molecular area decreased, and as the domain size remained nearly constant, the number of molecules per LC domain thus had to increase. In addition, the domain boundary length increased as the number of domains becamed higher.

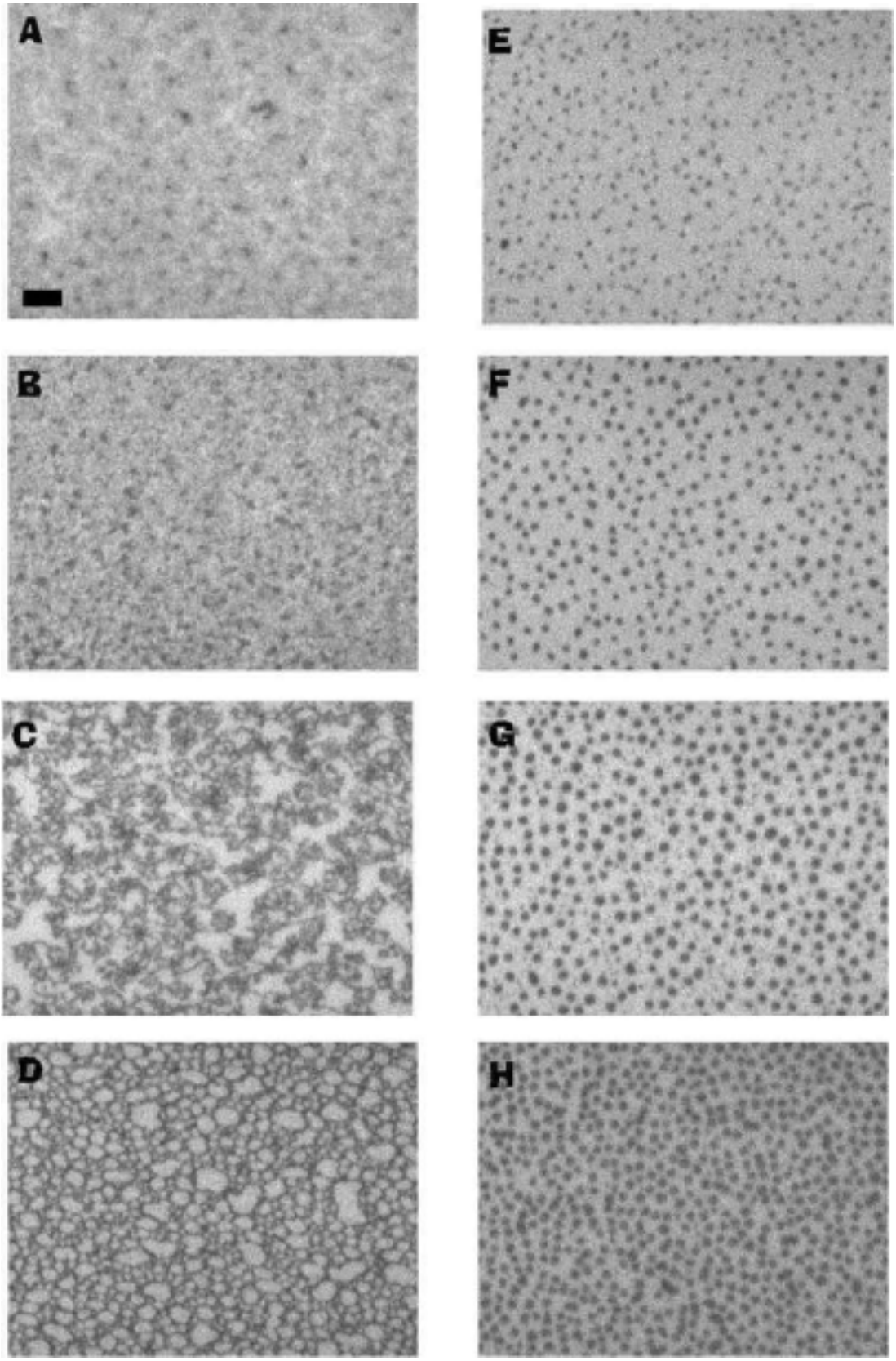


Figure 19. Fluorescence microscopy images of DPPC/cholesterol/NBD-PC (88:10:2) monolayers in absence (*panels A-D*) and presence (*panels E-H*) of CsA. Values of π were (*panel A*) 13.3 mN/m, (*panel B*) 18.8 mN/m, (*panel C*) 19.6 mN/m, (*panel D*) 30.5 mN/m, (*panel E*) 13.8 mN/m, (*panel F*) 17.7 mN/m, (*panel G*) 22.1 mN/m, and (*panel A*) 31.4 mN/m. Scale bar corresponds to 20 μm.

4.3. MEMBRANE INTERACTIONS OF CLOZAPINE, CHLORPROMAZINE, AND HALOPERIDOL (III)

In the following section the main emphasis is put on the comparison of the effects of an atypical neuroleptic CLZ, and conventional neuroleptics, CPZ and HPD. Classification of neuroleptics to conventional and atypical is based on the different (clinical) effects of these compounds on the negative and positive symptoms of schizophrenia, not on the chemical structure of the compound (Kapur and Remington, 2001).

Neuroleptic drug induced changes in the thermal phase behaviour of DPPC/brainPS MLVs

Progressive replacement of DPPC by brainPS broadened the main transition endotherm, and already at X_{PS}=0.05 a shoulder was clearly evident in the endotherm, thus indicating a phase separation. The main transition enthalpy decreased as the content of PS was increased. The T_m decreased gradually as X_{PS} was increased, but a small peak remained at ~41 ^{o}C. **(Fig. 20)**

The effects of CLZ, CPZ, and HPD on the thermal phase behaviour of DPPC/brainPS (95/5) MLVs were studied. In the presence of CPZ and HPD, the shoulder at ~41 ^{o}C was still evident, whereas CLZ abolished the phase separation. **(Fig. 21)** The pretransition temperature was decreased gradually by all three drugs, CLZ having the smallest effect. All three drugs abolished pretransition at low concentration (X_{CLZ}>0.03, X_{CPZ}>0.02 and X_{HPD}>0.02). The T_m was decreased by all three drugs, With CLZ having the smallest effect. The main transition enthalpy was increased by CPZ, and HPD caused a biphasic change in ΔH. CLZ had only very slight effects, except the minima observed at X_{CLZ}=0.05 (corresponding CLZ:PS ratio of 1:1). **(Fig. 22)**

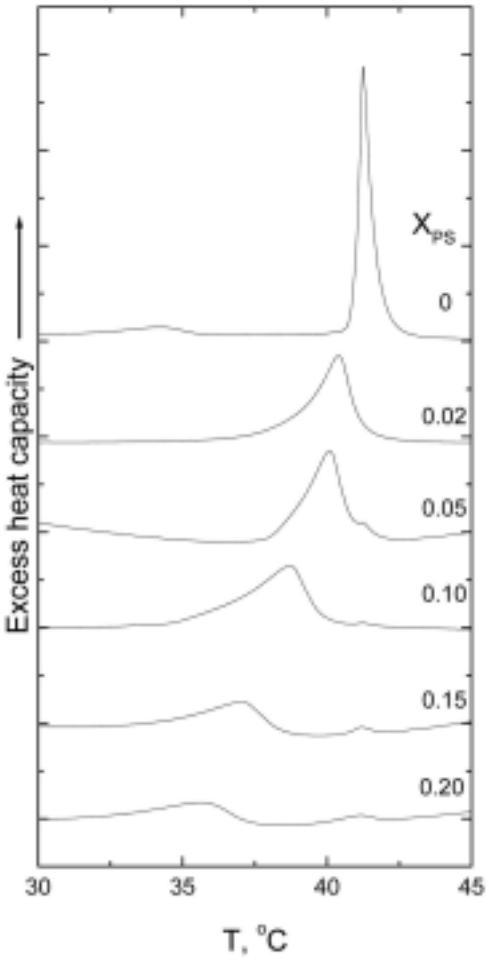


Figure 20. Effect of brainPS on the endotherms of DPPC MLVs.

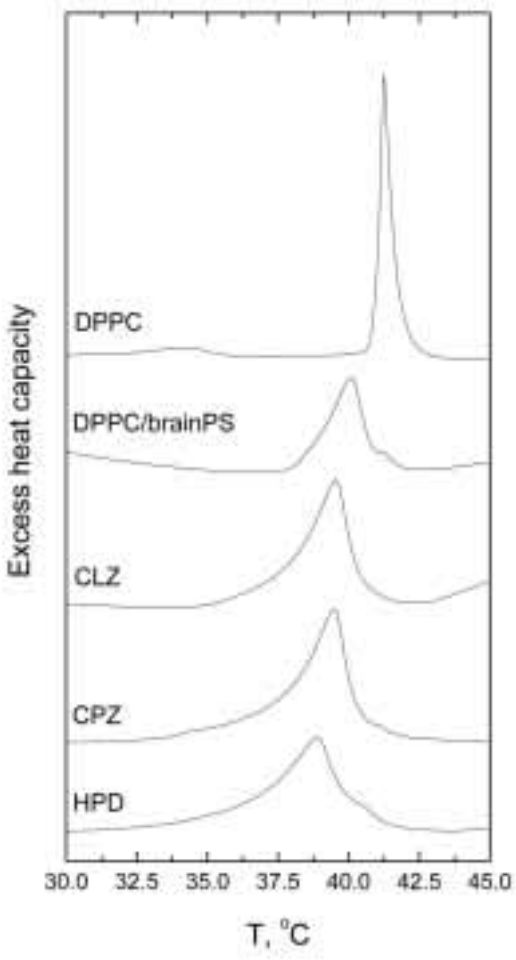


Figure 21. Effects of CLZ, CPZ, and HPD (X=0.05) on DPPC/brainPS (95/5) MLVs endotherms.

Binding of CLZ, CPZ, and HPD to lipid monolayers

The affinities of CLZ, CPZ, and HPD to zwitterionic eggPC and negatively charged brainPS monolayers were compared. CLZ produced the largest increase in $\Delta\pi$ in PC monolayers, and was able to penetrate into eggPC monolayer even at π_0>36 mN/m. In eggPC monolayers, the increase in $\Delta\pi$ caused CPZ and HPD was approx. half of that seen with CLZ. Additionally, the penetration of CPZ and HPD into eggPC monolayers was prevented at much lower lipid packing densities. In brainPS monolayers CPZ caused largest increase in $\Delta\pi$, and CLZ lowest. **(Fig. 23)**

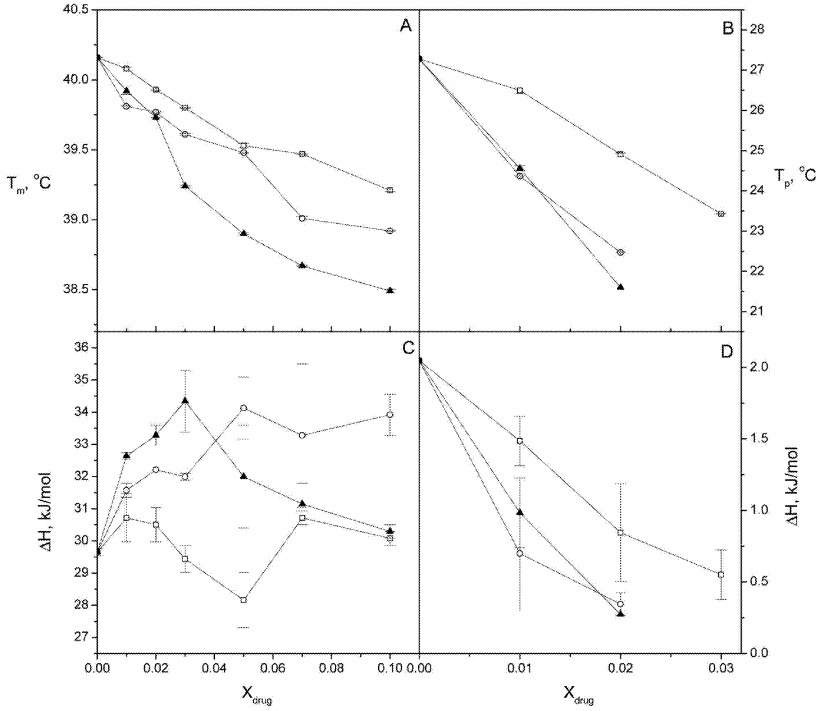


Figure 22. Effect of neurolepts CLZ (❑), CPZ (O), and HPD (▲) on DPPC/brainPS MLVs. *Panel A)* T_m, *panel B)* T_p, *panel C)* ΔH_m, and *panel D)* ΔH_p.

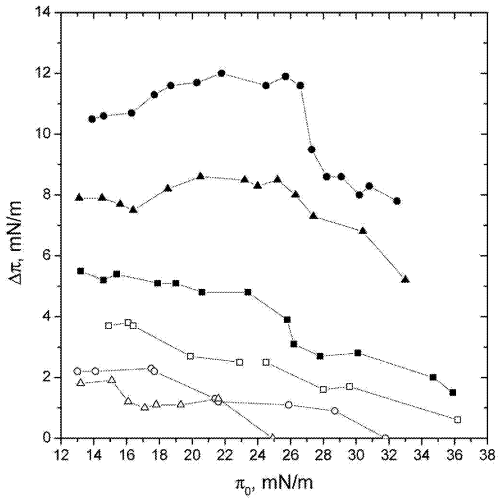


Figure 23. Changes in π of eggPC (open symbols) and brainPS (closed symbols) monolayers by addition of CLZ (❑, ■), CPZ (O,●), and HPD (Δ,▲).

The effect of CLZ, CPZ, and HPD on the compression isotherm of DPPC/brainPS (95/5) monolayers was also investigated. The plateau (corresponding to LE-LC coexistence region) was observed in absence and presence of the drugs. At $\pi < 10$ mN/m the isotherms for the three drugs were almost superimposable. However, the isotherms were clearly different from the pure lipid monolayer. At $\pi > 10$ mN/m the expansion of the monolayer was also evident for these drugs and CLZ caused the largest expansion. **(Fig. 24)**

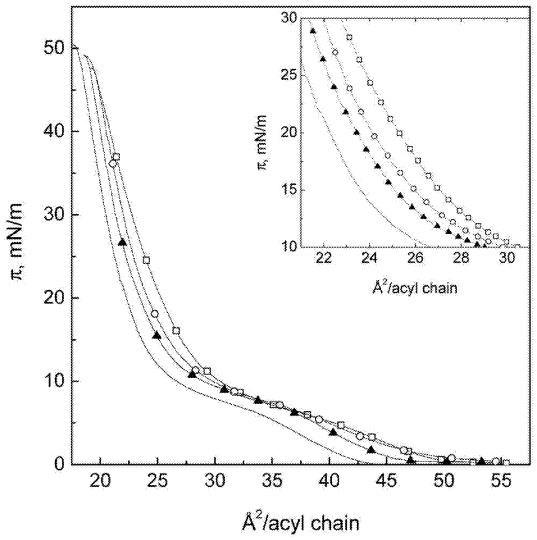


Figure 24. Effects of CLZ (❑), CPZ (O), and HPD (▲) on compression isotherms of DPPC/brainPS (95/5). Drug:lipid ratio was 5:100.

CLZ, CPZ, and HPD have different effects on the lipid monolayer organization

Changes in lipid domain morphology by CLZ, CPZ, and HPD were studied using DPPC/brainPS (95/5) monolayers. In the absence of drugs domains appeared at $\pi = 9$-10 mN/m, and the domains were quite spherical without any interfacial fine structure. As π was increased, the domain size increased, while the domain morphology remained similar. Visually it was evident that membrane lateral organization was pertubed by the drugs in order CPZ>HPD>CLZ. **(Fig. 25)**

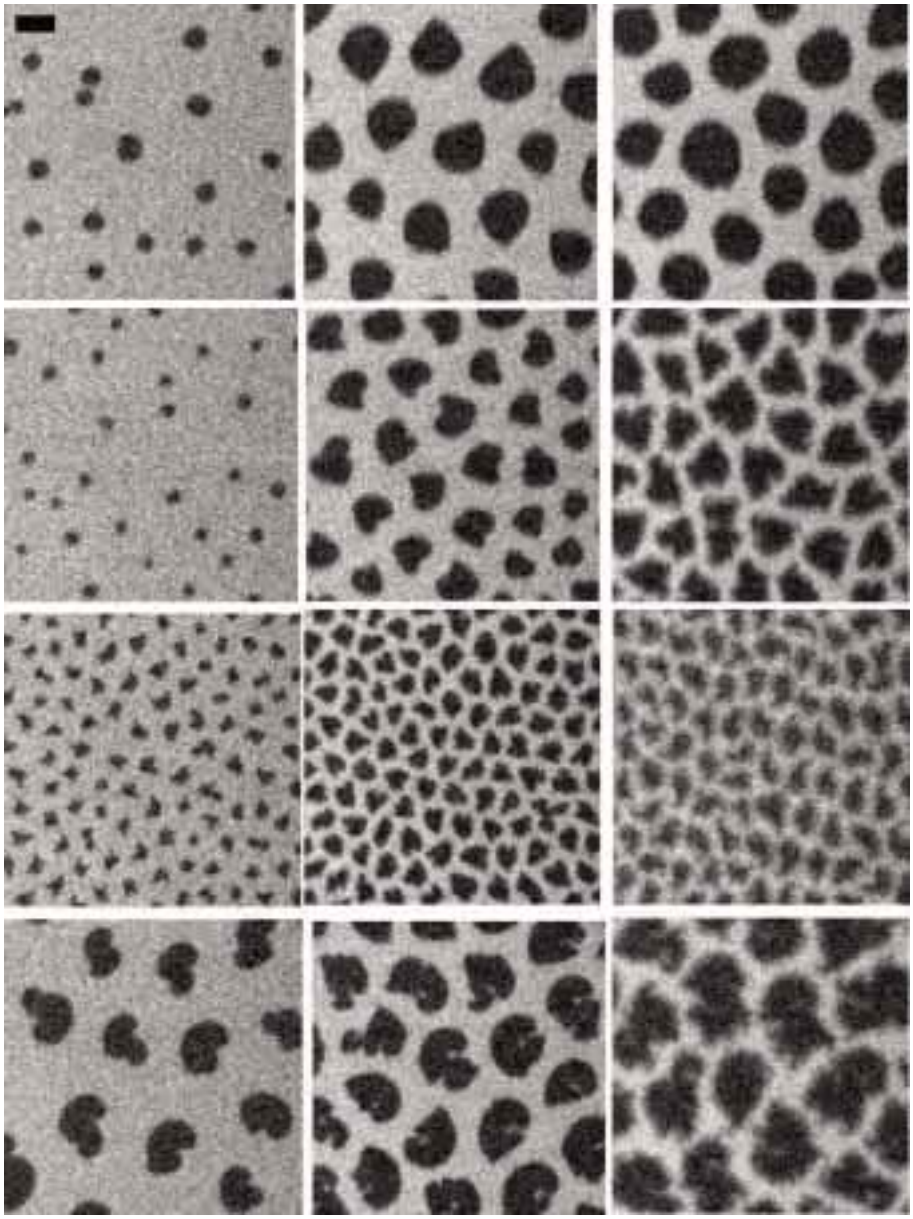


Figure 25. Neurolept induced changes in domain morphology of DPPC/brainPS monolayers. Horizontal rows (from top to bottom): no drug, CLZ, CPZ, and HPD. Vertical rows (from left to right): 15, 20, and 25 mN/m. Scale bar corresponds to 20 μm.

Overall the results indicate that all three drugs induced changes in the domain morphology and CLZ had the smallest effect. The changes produced by the drugs are clearly dependent on the compound, and are thus compound-specific.

4.4. SURFACE TENSION AND OSMOTIC PRESSURE (IV)

Fluorescence properties of Laurdan

The fluorescent naphtalene moiety of Laurdan possesses a dipole moment due to the partial charge separation between 2-dimethylamino and the 6-carbonyl residues and, upon excitation the dipole moment increases (Weber and Farris, 1979; Parasassi et al., 1998). Laurdan is virtually insoluble to water, thus the calculated GP value is obtained entirely from the fluorescent probes located into the membrane (Parasassi et al., 1993). The emission maxima of Laurdan in phospholipid membranes depends upon the phase state of the lipid, but is insensitive to headgroup, charge, and pH (Parasassi et al., 1991; 1998). In lipid membranes the fluorescent moiety of Laurdan is located at the level of glycerol backbones (Parasassi et al., 1998). The emission maxima is ~440 nm in gel phase and ~490 nm in fluid phase (Parasassi et al., 1998). The value for GP has been shown to assess the relaxation of the water molecules surrounding the fluorescent moiety of Laurdan in phospholipid membranes (Parasassi et al., 1991; 1998). The dipolar relaxation observed during the phospholipid phase transition and in the fluid phase is not due to the probe itself or reorientation, but is due to the water molecules penetrating to the glycerol backbone level (Parasassi et al., 1991; 1998). The generalized emission polarization (GP) value can be used to monitor water penetration into the bilayer, the higher the GP value, the lower the penetration (Parasassi et al., 1994; 1998).

Effects of betaine, choline chloride, and sucrose on γ

Several solutes such as NaCl (Adamson, 1990), polyols (Kaushik and Bhat, 1998), and many amino acid salts (Kita et al., 1994) have been reported to increase surface tension. Physically, a solute increasing surface tension of water will oppose an increase in the exposure of a hydrophobic surface to the aqueous phase. The effects of betaine, choline chloride, and sucrose on the Laurdan GP in bilayer membranes were measured. POPC LUVs were used as a model membrane. First, the effects of these three solutes on γ were measured at three osmotic pressures, 0.5, 1.0, and 2.0 osm/kg. γ was increased in the order of sucrose>betaine>choline chloride at all studied Π. **(Table II)** The largest increase was 2.8 mN/m by sucrose at Π= 2.0 osm/kg.

Table II. Increase in surface tension (Δγ) by betaine, choline chloride, and sucrose shown at increasing isoosmolar concentrations. The respective molal concentrations are shown in brackets.

	Osmolarity (osm/kg)		
	0.5	1.0	2.0
Betaine	0.17±0.09 (0.47)	0.42±0.15 (0.90)	0.80±0.16 (1.65)
Choline chloride	0±0.13 (0.29)	0.27±0.20 (0.59)	0.64±0.10 (1.22)
Sucrose	0.58±0.13 (0.47)	1.34±0.06 (0.91)	2.78±0.18 (1.70)

Effects of γ and Π on Laurdan GP

At equal osmotic pressures the GP value increased in the order sucrose>betaine>choline chloride **(Fig. 26)**, and the increments correlated with the observed increasing values for γ **(Table II)**. At 0.5 osm/kg choline chloride, a slight increase in GP without a change in surface tension was observed and could be explained by an increased osmotic pressure due to the solute. Importantly, as the ΔGP values were plotted against the change in Δγ a strong correlation was evident. **(Fig. 27)** A possible relationship between GP values and Δγ could be provided by the change in hydration as such, surface tension induced changes in membrane tension and membrane stiffness, and consequent changes in the free energy of membrane fluctuations. Attenuated water dynamics around the fluorescent moieties would likely cause increase the GP value.

In MLVs the bilayers are pushed into close proximity, which can change membrane dynamics. Also, in MLVs the access of osmolytes into the interlamellar space can be limited, thus causing osmotic gradients. In addition, MLVs in contact with excess solvent can spontaneously deplete small solute molecules from its interior by entropy-driven mechanisms (Diamant, 2002). For these reasons we used LUVs to exclude the impact of the above factors on the results. Freeze-thawing did not have any effect on the results.

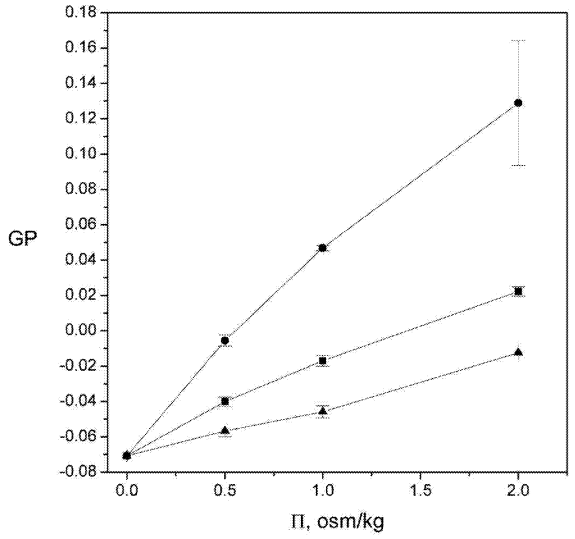


Figure 26. Changes in Laurdan GP by betaine (■), choline chloride (▲), and sucrose (●) at equal Π.

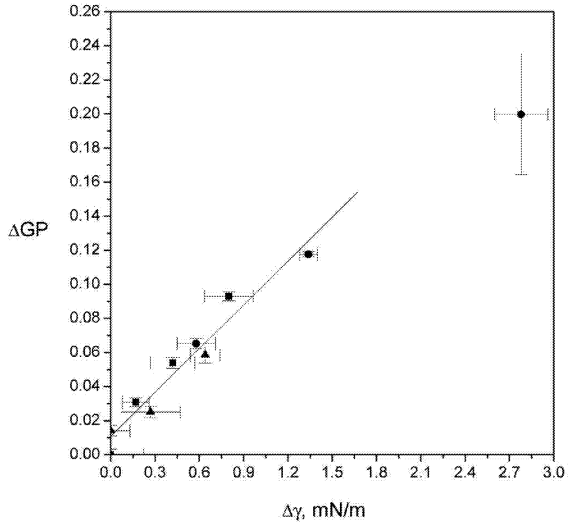


Figure 27. Correlation between Laurdan GP and Δγ induced by betaine (■), choline chloride (▲), and sucrose (●).

To conclude, if the osmotic pressure would be the dominant factor modulating hydration of the membrane interface, no differences would be expected between these solutes at equal osmotic pressures. However, at equal osmotic pressures different

solutes clearly show different effects on GP, whereas GP values fall on single curve when plotted against $\Delta\gamma$.

Surface tension for water decreases with increasing temperature (Weast, 1979). Laurdan GP values for POPC LUVs in the temperature range from 5 to 50 °C in the absence of kosmotropes were measured as an independent test for the correlation between Laurdan GP and γ. The GP value decreased with increasing T. **(Fig. 28)** Measurement of GP is essentially ratiometric and therefore only negligible influence due to thermal deactivation of the excited state of the fluorophore can be expected. This suggests that the temperature dependent changes in GP values for fluid, unsaturated POPC LUVs could also be explained by the temperature dependence of γ. The data for GP measured at 25 °C as a function of γ (modulated by solutes) coincided very well with GP vs. γ (by T) data thus suggesting a common mechanistic basis. **(Fig. 28)**

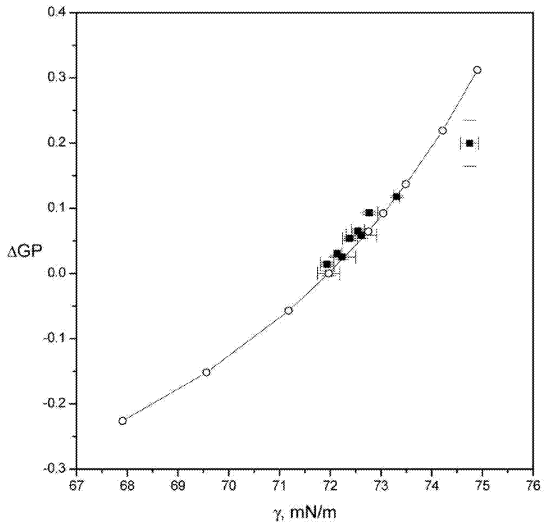


Figure 28. Correlation between Laurdan GP and $\Delta\gamma$. Changes in γ were induced by temperature (O) or by osmolytes (■).

5. DISCUSSION

When considering the importance of drug-lipid interactions in respect to pharmacology, it should be considered that most *in vivo* processess do include an interaction with a lipid membrane. Accordingly, a detailed knowledge of mechanisms and consequences of drug-lipid interactions is likely to enhance our understanding on the mechanisms of action, and/or adverse effects, and the pharmacokinetic behaviour of drugs. The drug-lipid interactions can lead to many different events, including the prevention of the access of drug to active (target) site, accumulation of the drugs to the membrane, conformational change in the drug and in the phospholipids, changes in lipid dynamics, membrane thickness, permeability, cooperativity, surface potential, and hydration (Fruttero, 2001). In this respect, also the knowledge of factors affecting the above mentioned membrane properties is important.

Conventionally drugs are *a priori* assumed to exert their action in cells by more or less specific binding to sites in proteins. However, some compounds, such as amphotericin B (Andreoli, 1974; de Kruijff and Demel, 1974; Gallis et al., 1990; Cohen, 1992; Barwicz and Tancrède, 1997), and cytotoxic peptides, e.g. cecropins, defensins, and magainins (Bechinger, 1997) are known to exert their pharmacological effects by lipid-mediated mechanisms without a known involvement of specific proteins. In this regard the rich scale of different phases, i.e. membranes with distinct physicochemical properties, exhibited by different lipids is of interest (Kinnunen and Laggner, 1991). There is experimental evidence indicating a correlation between the physical state, i.e. the phase state of cellular membranes, determined by their lipid composition, and the physiological state of the cell (Kinnunen, 1991; Kinnunen, 1996). There is no reason to believe that the modulation of specific properties of the lipid bilayer would be limited to these compounds but it could contribute both to the therapeutic mechanism as well as side effects of membrane-partitioning compounds in general.

To this end, a large variety of structurally dissimilar drugs are hydrophobic or amphiphilic and readily partition into lipid membranes (Fischer et al., 1998; Schreier et al., 2000), good examples being provided by tacrine (Lehtonen et al., 1996b), doxorubicin (Mustonen and Kinnunen, 1991; 1993; Mustonen et al., 1993), and

cyclosporin A (O´Leary et al., 1986; Wiedmann et al., 1990). Drugs may also modulate peripheral lipid-protein interactions as shown for chlorpromazine (Ito et al., 1983), doxorubicin (Mustonen and Kinnunen, 1991), lidocaine, and gentamycin (Jutila et al., 1998).

5.1 DETERMINANTS OF DRUG-LIPID INTERACTIONS: MEMBRANE PROPERTIES

Acidic phospholipids

Doxorubicin was used as a model drug to study the effect of acidic phospholipid content on the kinetics of membrane association of a drug. Although exact values for binding constants cannot be calculated from the resonance energy transfer data the difference in the values for $t_{1/2}$ of binding of doxorubicin onto membranes readily translates into an approx. 1000-fold enhancement in the binding rate in the presence of the acidic lipid. Reversal of the fast component in the fluorescence decay by increasing [NaCl] and [$CaCl_2$] clearly indicate this process being mediated by electrostatic attraction. The large differences in the kinetics of membrane association of doxorubicin could be important in the cellular distribution (or blood-tissue distribution) of this drug.

The irregular behaviour of $t_{1/2}$ of the electrostatic binding of doxorubicin at low X_{PG} (0.04-0.10) suggests membrane association of this drug to be dependent on the initial distribution of charges in the membrane (*see below*). Also, the 1000-fold difference in the half-times of the binding can be used to assess the membrane lateral heterogeneity before the binding of doxorubicin.

The monolayer measurements indicate a clear difference in the modes of interaction of CLZ, CPZ, and HPD with lipid monolayers. Among the three neuroleptics studied, CLZ had the strongest affinity towards membranes composed by neutral lipids, whereas CPZ and HPD had higher affinity towards negatively charged lipids. The ratio of changes in $\Delta\pi$ in PC/PS monolayers was clearly different for CLZ when compared to CPZ and HPD. **(Table III)**

Table III. Increase in π by CLZ, CPZ, and HPD in brainPS and eggPC monolayers at π_0= 15, 20, and 25 mN/m. The lower panel shows $\Delta\pi_{PS/PC}$, the ratio of Δπ in brainPS and eggPC monolayers. All π values are expressed as mN/m.

π_0		CLZ Δπ	CPZ Δπ	HPD Δπ
15	PC	3.8	2.2	1.9
20	PC	2.7	1.7	1.2
25	PC	1.6	0.5	0
15	PS	5.4	10.6	7.8
20	PS	4.9	11.7	8.5
25	PS	2.8	8.4	6.9

PC/PS (π_0)	CLZ	CPZ	HPD
15	0.7	0.21	0.24
20	0.55	0.15	0.14
25	0.57	0.06	0

The number of compounds studied was small, hence it would be of interest to study whether similar differences in the ratio of Δπ of PC/PS would be observed also for other atypical and conventional neuroleptics. CLZ had the strongest impact on the compression isotherms of DPPC/brainPS monolayers. At higher surface pressures the average molecular area was increased in the order of CLZ>CPZ>HPD. The effects of these drugs on the average molecular area were increased in the same order as Δπ in eggPC monolayers. This suggests that the increase in molecular area was mainly due to the hydrophobic interaction between drugs and lipid monolayer. The electrostatic interaction between drug and lipid occurs at the headgroup region of the lipid membrane. The intercalation of the drug between lipid acyl chains after electrostatic binding is most likely compound-specific. The binding of CPZ has been shown to induce *gauche* conformation in gel phase saturated PC/PS membranes without intercalation of the drug between acyl chains (Nerdal et al., 2000). The work of insertion of CPZ between acyl chains in acidic monolayers is highly dependent on the acyl chain length and saturation (Agasøster and Holmsen, 2001). The work of insertion decreases by $15*10^{-20}$ J/CPZ by adding one double bond into acyl chain and by $5*10^{-20}$ J/CPZ by shorthening the acyl chain by two carbons (Agasøster and

Holmsen, 2001). For CPZ the intercalation of the drug into DPPC/brainPS monolayers can be expected to be lower than for pure unsaturated brainPS monolayers. Thus, at least for CPZ, the increase in average molecular area of DPPC/brainPS monolayers is likely to be caused by (purely) electrostatic interactions.

The drop in $\Delta\pi$ at ~25-26 mN/m observed for each drug in PS monolayers is likely reflecting a membrane dependent effect rather than drug-dependent. A possible explanation for the observed effect could be deprotonation of PS at higher lipid packing densities, leading to formation of hydrogen bonding network between PS headgroups (Boggs, 1987) and decreased net negative charge of the monolayer. These results indicated that compared to two conventional neuroleptics, CPZ and HPD, the atypical neuroleptic drug CLZ had a different effect on the thermal phase behaviour of DPPC/brainPS.

Reconstituted dopamine D_2-receptor requires a mixture of PC, PE, and PS for restoration of its ligand binding (Srivastava et al., 1987), with PS being particularly important. The depletion of PS from dopamine D_2-receptors could thus diminish the ligand affinity. HPD has been reported to reverse PS induced inhibition of phosphatidylinositol formation (Bonetti et al., 1985). Strong interaction between brainPS and neuroleptic drugs could detach PS from neurotransmitter receptors, e.g. dopamine D_2-receptor, thus leading to altered function of the protein, as shown for the inhibition of cytochrome *c* oxidase by doxorubicin (Goormaghtigh et al., 1982).

Membrane lateral heterogeneity

The fast binding of doxorubicin observed at T<34 ^{o}C reveals the formation of microdomains enriched in PG (i.e. with local X_{PG}>0.04), having increased negative surface density charge. The lack of the slow hydrophobicity driven binding could reflect the tight packing of the gel state acyl chains preventing the intercalation of doxorubicin into the bilayer. At T>34 ^{0}C also the slow hydrophobicity driven membrane association of doxorubicin becomes evident. This is likely to result from the formation of ´fluid´ domains in the bilayer (Marsh et al., 1977; Doniach, 1978; Freire and Biltonen, 1978; Pedersen et al., 1996; Jutila and Kinnunen, 1997). The emergence of ´fluid´ domains in gel bulk is consistent with the enhanced hydrophobic

binding of doxorubicin to the electrically neutral DPPC/PPDPC LUVs (containing no acidic phospholipid) upon exceeding T≈33 ^{0}C. However, as the slow binding process is observed for the DPPC/PPDPC LUVs already within the temperature range of 30 to 33 ^{0}C it is possible that the lack of the slow component for DPPC/PPDPG LUVs at temperatures <34 ^{0}C is apparent only, as follows. For the DPPC/PPDPG liposomes the slow component would not be seen as a large fraction of the drug is already bound to the clustered acidic phospholipid, thus reducing the concentration of doxorubicin remaining in the solution after the fast, electrostatically driven membrane association of the drug is complete. Also, as PPDPGs are mainly segregated to domains, binding of doxorubicin to DPPC matrix does not influence on the fluorescence emission. At T>34 ^{0}C also the decrease in RFI as a function of temperature becomes augmented in DPPC/PPDPG LUVs. In other words, *n* (the number of PPDPG molecules encriched in clusters) diminishes already when approaching T=34 ^{0}C. Upon exceeding T=34 ^{o}C, another process causing decrease in *n* becomes effective. The interpretation of the appearance of the slow fluorescence decay at T=34 ^{0}C as the formation of ´fluid´ domains in the gel state bilayer is supported by the DSC measurements which revealed onset of the main phase transition endotherm at 34 ^{0}C in the DPPC/PPDPG LUVs. Simultaneously with increasing temperature in the range from 34 to 38 ^{0}C, the PG enriched clusters seem to be diminishing either in size or number as the RA of the fast fluorescence decay is steeply attenuated.

The fast fluorescence decay for DPPC:PPDPG (97:3, mol/mol) LUVs is not observed at T> 37 ^{0}C. This temperature coincides with the plateau in I_e/I_m observed in steady-state measurements. The apparent lack of clusters of PPDPG is revealed by the absence of the fast, electrostatic component in doxorubicin binding above 37 ^{0}C. This is intriguing as steady-state measurements reveal highly efficient excimer formation also in the temperature range of 38 to 41 ^{0}C. Previously, PPDPC has been suggested to preferentially accommodate within the interface between gel and ´fluid´ domains of DMPC (Jutila and Kinnunen, 1997). Similar behaviour for PPDPG in the melting of DPPC matrix would be compatible with the present data. Accordingly, the characteristics of the fast component at 37 ^{0}C (i.e. minimum in both $t_{1/2}$ and amplitude) would result from a change in the dimensionality of the lateral arrangement of PPDPG from 2-D (clusters) to pseudo 1-D (arrays), the latter being

formed in the domain boundaries. For the latter efficient excimer formation is still evident. Importantly, already small amounts of doxorubicin associated with the PPDPGs concentrated in these 1-D arrays residing within the domain boundaries will strongly diminish the effective charge density and yield a small RA for the process, even if the initial affinity would be high.

Alternative explanation is that the size of the PPDPG clusters decreases progressively with temperature, and reaching at T≥37 ^{0}C the domain size no longer having sufficient number of charges per unit surface area to provide the sites for fast binding of doxorubicin. However, the latter mechanism does not explain the fact that $t_{1/2}$ has a minimum at 37 ^{0}C. Also, the area of ´fluid´ domains at 38 ^{0}C, for instance, should be 75 % of the total liposome surface area in order to reduce the concentration of the probe below X_{PG} 0.04 and, accordingly, to abolish the fast binding. This seems unlikely because the area under the endotherms up to 38 ^{0}C corresponds to only approx. 10 % of the total enthalpy of the transition.

Thus, the first mechanism is considered to be most plausible. The driving force for the enrichment of PPDPG into the boundaries between ´fluid´ and gel domains in the temperature range of 34 to 38 ^{0}C can be rationalized as follows (Jutila and Kinnunen, 1997). As a perturbating substitutional impurity PPDPG is expelled from the gel state DPPC matrix leading to segregation of PPDPG into domains as shown for PPDPC (Galla and Hartmann, 1980; Somerharju et al., 1985; Hresko et al., 1986; Jones and Lentz, 1986). Upon the gel-to-fluid transition thickness of the DPPC bilayers is reduced by approx. 4.5 Å, from 23 to 18.5 Å (Chapman et al., 1967; Lewis and Engelman, 1983). Hydrophobic mismatch has been shown to promote the clustering of PPDPC in liquid crystalline bilayers of thickness < 20.7 Å (Lehtonen et al., 1996a). The effective length of PPDPG should equal to that of PPDPC. We can thus expect PPDPG to be expelled to some extent also from the ´fluid´ domains below T_m, eventhough fluid domains exhibit higher compressibility (Ali et al., 1998; Li et al., 2000). Accordingly, preferential partitioning of the probe into the domain boundary can be anticipated. The compressibility of fluid phase membrane is higher than in gel phase, and thus a higher content of PPDPG is likely to be present in fluid domains than in gel domains.

Taken together, these measurements provide evidence for the formation of ´fluid´ domains in the gel state matrix to begin at a temperature well below the maximum of the main transition endotherm determined by DSC. The comparison of the stopped-flow data with values of I_e/I_m derived from steady-state fluorescence measurements provide further evidence for the enrichment of the fluorescent probe, PPDPG into the interface separating gel state and fluid domains in the coexistence region, present in the course of the transition. These data lend further support to previous results indicating the length of the interfacial boundary to have a maximum well below the main endotherm, in this case at approx. 3.5 degrees below the DSC peak at 40.4 ^{0}C. The fluorescence data indicate lack of ´fluid´/gel phase boundaries in the range from 38 ^{0}C to 43.7 ^{0}C. Yet, as the peak in heat capacity is observed at 40.4 ^{0}C the molecular mechanisms underlying the enthalpy changes in this temperature range and the nature of the implied ´intermediate´ phase pose an intriguing problem. Regarding the latter, it is possible that in this intermediate phase the chain order and lattice translational and positional order are uncoupled, similarly to the liquid-ordered phase present in the phospholipid-cholesterol phase diagram (Vist, 1984; Ipsen et al., 1987; Vist and Davis, 1990).

The uncoupling of lattice melting and acyl chain melting was suggested for lipid bilayers of long-chain (17-20 carbons) saturated diacyl PCs (Nielsen et al., 1996). The uncoupling of lattice and acyl chain melting was predicted from computer simulations based on the experimental DSC data. In this model the sub-main transition ($T_p<T_{sub-main}<T_m$) would represent chain-ordered/crystalline-ordered to chain-ordered/crystalline-disordered transition, i.e. lattice melting, and in main transition (T_m) the membrane would proceed from chain-ordered/crystalline-disordered to chain-disordered/crystalline-disordered, i.e. acyl chain melting. However, the strong thermal fluctuations near the main transition of DPPC should vanish the sub-main transition (Nielsen et al., 1996).

Lipid lateral packing and cholesterol

In lipid mixtures cholesterol can cause the formation of ´liquid-ordered phase´, which possess properties from both gel and fluid phase, i.e. cholesterol decreases the acyl chain motion of phospholipids, but the lateral diffusion remains high. The phase

behaviour of cholesterol-phospholipid mixture is complex (Vist, 1984; Ipsen et al., 1987; 1989; Vist and Davis, 1990; Feigenson and Buboltz, 2001). Membrane defects, such as domain boundaries are more permeable than the bulk phase, and the increased membrane permeability is linked to the length of these boundaries (Cruzeiro-Hansson and Mouritsen, 1988). At low mole fractions cholesterol has been suggested to preferentially partition into domain boundaries (Weis and McConnell, 1985). This could explain the permeability enhancing effect of cholesterol when present at low concentration (Carruthers and Melchior, 1983; Corvera et al., 1992; Zuckermann et al., 1993). However, at higher concentration of cholesterol, the increased lateral packing decreases the free volume of membrane, resulting in decreased permeability (Demel et al., 1972; Almeida et al., 1992; Yan and Eisenthal, 2000). The nature of cholesterol-phospholipid interactions, and miscibility of cholesterol in membrane depends on the structure of lipid headgroup and acyl chains and on the temperature and phase state of lipid bilayer (McMullen et al., 1993; Smaby et al., 1994; McMullen and McElhaney, 1997).

The biphasic, packing density dependent interaction of CsA with the phospholipid monolayer reveals cholesterol to decrease the penetration of CsA into the lipid monolayer in a surface pressure dependent manner. Cholesterol increases lateral packing density of PC monolayers and decreases the free volume of the hydrophobic part of the monolayer causing phospholipid condensation (Gershfeld and Pagano, 1972; Smaby et al., 1994). Obviously, this would impede the penetration of CsA into the lipid. Cholesterol increases membrane lateral compressibility (Rand et al., 1980; Smaby et al., 1997). Incorporation of identical amount of CsA molecules in PC membranes in absence and presence of cholesterol, thus causes a larger increase in $\Delta\pi$ for cholesterol containing membranes. As cholesterol decreases $\Delta\pi$ after addition of CsA on the subphase, it is obvious that cholesterol decreases membrane partitioning of CsA. Yet, also more specific lipid-drug interactions could be involved, and could reflect a pressure-induced change in the conformation and/or orientation of CsA. In this case, the conformation and/or orientation of CsA in the membrane would also be sensitive to cholesterol.

Membrane dipole potential (Ψ) arises from the oriented lipids (dipoles) in the membrane, in a complex manner as the contributions of bound water molecules, headgroups, glycerol ester groups, and the terminal methyl groups of acyl chain are considered (Brockman, 1994; 1999). Ψ originates from the overbalanced dipole moments of phospholipids by the oriented water molecules (Chiu et al., 1995; Marrink et al., 1996). The magnitude of electric fields across the membrane interface can be up to 10^7 V/cm due to Ψ, and such strong electric fields can be of importance in the sructure and function of biomembranes (Brockman, 1994). Differences in the dipole potentials between gel and fluid phases is important determinant of the size, shape, and lateral arrangement of lipid domains in monolayers (Brockman, 1994). Also, Ψ effects on the transport rate of hydrophobic anions and cations across the membrane (Brockman, 1994; 1999). The binding of Ψ decreasing compounds is augmented by incorporating Ψ increasing compounds into membrane. CsA decreases Ψ (Söderlund et al., *unpublished observation*), and cholesterol increases Ψ. However, as cholesterol decreases membrane binding of CsA it is evident that for membrane partitioning of CsA, other factors than Ψ are more important.

Cyclosporin A has been shown to make cholesterol in low-density lipoproteins more susceptible to oxidation (Apanay et al., 1994) although the molecular mechanism(s) is unclear. In this respect, the CsA-cholesterol interactions and effects of CsA on the intrinsic dynamics of low-density lipoprotein could provide information on this issue.

Superlattices and irregular dependence of X_{PG} vs. $t_{1/2}$

The irregular dependence of $t_{1/2}$ vs. X_{PG} at X_{PG}= 0.04-0.10, and the absence of fast fluorescence decay at X_{PG}<0.04 could be explained by the formation of superlattices of acidic phospholipids in the liquid crystalline membranes. Lack of the fast decay at X_{PG}< 0.04 is not only apparent as RA of this process does not extrapolate to zero at X_{PG}=0. In brief, due to coulombic repulsion the distribution of the deprotonated PGs can be readily expected to have a free energy minimum when the separating distances are maximal (Träuble, 1977; Denisov et al., 1998). At certain X_{PG} the surface charge density becomes high enough to cause the rapid binding of doxorubicin. The effective net negative surface charge density does not increase monotonously with X_{PG} but is a step function due to the higher surface charge densities. As a consequence, there is a

sharp threshold in X_{PG} above which the coulombic attraction of the drug is greatly enhanced. At X_{PG}=0.037 maximal separation of charges is achieved when PG molecules are each separated by 5 rows of PC. However, when X_{PG} exceeds 0.037 the added PG molecules must be accommodated in the membrane at interstitial sites within the original superlattice. Accordingly, there is a sharp local increment in net negative surface charge density. Further increase in X_{PG} necessitates the formation of a denser lattice. A maximum in $t_{1/2}$ observed at X_{PG}=0.065. This is in accordance with previously predicted hexagonal superlattice (Virtanen et al., 1988), yielding the free energy minimum for this assembly. Importantly, for this lattice the average charge density is smaller than in the intermediate distribution pattern between X_{PG}=0.037 and 0.065. The free volume in fluid membranes has been shown to be lower at critical mole fractions where regular distribution is observed (Chong et al., 1994). Accordingly, the rate of the membrane association of doxorubicin should decrease, as observed.

In parallel with increase in X_{PG} also the affinity of the bilayer surface for protons increases progressively thus decreasing the degree of the dissociation of the phosphate moiety of PG (Träuble, 1977), with concomitant reduction in surface charge density. In addition, intermolecular hydrogen bonding between protonated and deprotonated PG headgroups becomes possible (Boggs, 1987), which necessarily will also influence the ordering in the binary lipid alloy and compete with the interaction of doxorubicin with the deprotonated acidic phospholipid. The above is in keeping with the membrane binding of cytochrome c, another cationic ligand, becoming slower with increasing X_{PG} in the liposomes (Subramanian et al., 1998). Obviously, the above processes will also affect the rate of binding of doxorubicin to the liposomes.

5.2. CONSEQUENCES OF DRUG-LIPID INTERACTIONS: DRUG INDUCED CHANGES IN MEMBRANES

Membrane lateral organization – thermal phase behaviour

The thermal phase behavior of DMPC/cholesterol (10:1, mol/mol) liposomes as a function of X_{CsA} is complex. A likely explanation to these data could be provided by the same principles as forwarded for tacrine induced changes in the thermal behavior of dimyristoylphosphatidic acid (Lehtonen et al., 1996b). The latter results were interpreted in terms of formation of regular superstructured regions in the bilayer at well-defined drug:phospholipid ratios. In principle, all systems organize so as to minimize their free energy. In a bilayer composite alloy this may require its components to respond to modified lipid composition by changes in organization. This is observed by the alterations observed in the main transition enthalpy at CsA:cholesterol ratios of 3:10 and 1:2 (X_{CsA}=0.03 and 0.05, respectively). The regular organization of fluid lipid membranes in hexagonal superlattices has been shown to occur in many different membranes (Somerharju et al., 1985; Kinnunen et al., 1987; Virtanen et al., 1988; Tang and Chong, 1992; Sugar et al., 1994; Chong et al., 1994; Tang et al., 1995; Cheng et al., 1997; Liu et al., 1997). The observed minima in DMPC/cholesterol/CsA mixtures could be resulting from superlattices forming in the presence of CsA, similarly as suggested for another drug, tacrine (Lehtonen et al., 1996b). CsA could exert similar effects although not identical to those of cholesterol (Wiedmann et al., 1990).

Due to the negative charge of brainPS, these lipids would prefer maximum average distance between the headgroups. The non-saturated acyl chains of brainPS disturb the packing of DPPC acyl chains. In absence of negative charge in PS, the brainPS would most likely be phase separated from DPPC matrix as the unsaturated acyl chains would be expelled from saturated DPPC matrix. Due to the electrostatic repulsion between PS headgroups, the complete phase separation is energetically unfavourable. However, at DPPC/brainPS ratio of 95:5, it seems that certain fraction

of the membrane is devoid of brainPS, as evidenced by the phase separation peak at ~41.3 ^{o}C, corresponding to T_m of pure DPPC.

The phase separation in DPPC/brainPS (95/5) MLVs is also readily observed in the presence of CPZ or HPD. This suggests that these two drugs are not able to efficiently partition into pure DPPC domains, whereas CLZ abolishes the phase separation. The effect of CLZ is likely to be due to its stronger hydrophobic interaction with membranes when compared to CPZ and HPD. The data clearly shows that atypical neuroleptic CLZ has different impact on the membrane lateral organization than conventional neuroleptics, CPZ and HPD.

Membrane lateral organization - domain morphology

Fluorescence microscopy results with CsA indicated, that changes in the domain morphology are dependent on the membrane lipid composition. CsA induced changes in DPPC monolayers suggest CsA to stabilize domain interfaces, as the diffuse domain boundaries at higher π become sharp. The present results show that the interaction of CsA with membranes containing cholesterol are much more complex than that revealed in previous studies with pure PC bilayers (Wiedmann et al., 1990).

The important question on the drug induced changes in membrane lateral heterogeneity is whether the changes are drug-specific or non-specific. The observation that all the three neuroleptic drugs altered the domain morphology but in a different manner, indicates that the effects were drug-specific.

DSC is not as sensitive technique as fluorescence microscopy, and thus higher concentrations of both lipids and drugs are needed. Also, the direct comparison of the different membrane models, liposomes and monolayers, is ambiguous, but all studied drugs (CsA, CLZ, CPZ, and HPD) changed the membrane lateral heterogeneity in both liposomal (DSC) and monolayer (fluorescence microscopy) model membrane systems.

Drug-induced changes in domain morphology: physiological significance

This is the first time that drugs have been observed to induce changes in domain morphology *in vitro*. Previously, using Monte Carlo computer-simulations (*in silico*) several membrane-perturbating drugs were found to accumulate in gel-fluid domain boundaries, inducing alterations in the membrane lateral heterogeneity (domain size and morphology) at the molecular level (Jørgensen et al., 1991a; 1991b; 1993; Sabra et al., 1995; 1996). The accumulation of drugs at the domain interfaces leads to a much higher local concentration of the drug than the bulk drug concentration in the membrane (Jørgensen et al., 1993). A similar accumulation could also give rise to local changes at the lipid-protein interface, leading to altered protein conformation and function (Jørgensen et al., 1993).

The finite lifetime of the observed domains in the simulations are controlled by line tension (Mouritsen and Kinnunen, 1996). The determinants of the domain shape in lipid monolayers have been a subject of intense research (Weis, 1991) and dipole repulsion and line tension are considered to be the dominant factors (Möhwald, 1995; Brockman, 1999). Line tension is a 1-D pressure and the dipole repulsion results from noncompensated molecular dipole moments (Möhwald, 1995; Brockman, 1999). The line tension tends to make domains compact and circular with minimal domain boundary length, while dipole repulsion have the opposite effect (Brockmann, 1999). Even though the domain sizes observed in lipid monolayers are usually larger than individual cells, the determinants of the domain morphology may also contribute to the nanoscale organization of the lipid membranes.

In giant unilamellar vesicles, 2-D domain formation can cause changes in 3-D vesicle morphology, such as budding and vesiculation (Jülicher and Lipowsky, 1993; Lipowsky, 1993; Sackmann and Feder, 1995; Holopainen et al., 2000b). The shape of a vesicle depends on the relative area of the inner and outer leaflets, area-to-volume ratio of the vesicle, shape differences between the lipids in the inner and outer leaflets, and on the lateral distribution of lipids on the vesicle membrane (Kas and Sackmann, 1991; Döbereiner et al., 1993; Jülicher and Lipowsky, 1993). The formation of domains can have a significant effect on the local (3-D) shape of the membrane. The shape taken up by the domain depends on the balance between bending energy and

line tension of the domain (Lipowsky, 1992; Jülicher and Lipowsky, 1993). The membrane surface can reduce its energy by forming a bud with the domain boundary in its base, and provided that the line tension times the boundary length is equal or greater than the bending energy, the bud can pinch off from the vesicle (Bradley et a., 1999). Thus, the accumulation of drugs at domain boundaries, and their effect on the line tension could also cause changes in the 3-D shape of a lipid membrane. This provides a potential mechanism for the drugs to modulate cellular functions involving a formation of lipid vesicles. One of the side-effects of CsA is nephrotoxicity, which histopathological findings include tubular epithelium and myocyte vacualization (Randhawa et al., 1993). These vacuoles (vesicles budding off) are typically fine and isometric in nature (Randhawa et al., 1993). The molecular level mechanisms of this vacualization remains unknown, but the above discussed mechanism could be involved.

A two-dimensional reticular structure formed by gel phase leading to the formation of separated fluid domains has been suggested to occur in lipid bilayers (Vaz et al., 1989; 1990). This type of two-dimensional ordering can reduce the rate of bimolecular processes and enzyme catalyzed reactions, if substrates and products are both confined into fluid, disconnected domains (Melo et al., 1992). In DPPC/cholesterol monolayers CsA destroyed the reticular network of gel phase to disconnected small gel domains. This change in lipid organization could affect the rate of some cellular functions occuring at the membrane surface.

The clinical relevance of the current results are unclear at the moment. However, it should be emphasized that the blood concentration of CsA *in vivo* is in the range of ~80-250 nM (Dollery, 1999b), and the calculated CsA concentration in the subphase in the monolayer experiments was 37.5 nM. Also, for the neuroleptic drugs, the subphase concentration in fluorescence microscopy measurements was low ~34 nM, which is much below the dissociation constant (0.1-10 μM) of these drugs from different receptors and their subtypes (Brody et al., 1998). In addition, the used neuroleptic drug concentrations were in the range of their therapeutic plasma concentrations (Dollery, 1991; Spina et al., 2000). Interestingly, it was recently reported that a correlation exists between surfactant or polymer induced changes in

the lipid monolayer organization (in μm scale) and the effect of the surfactant or polymer on the cell growth and viability (Yang et al., 2001).

5.3. INTERFACIAL DYNAMICS OF LIPID BILAYER: effects of γ and Π

The results with Laurdan GP are readily understood when considered within the framework of membrane lateral pressure profile. The surface tension exerts its effect in the thin interfacial region accomodating the fluorescent moiety of Laurdan, i.e. at the level of phospholipid glycerol backbone within the dynamic interface between water and hydrocarbon phase. As pointed out by Cantor (1997a) the magnitude of the pressures prevailing in membranes are considerable, corresponding to bulk pressures of many hundreds of atmospheric pressures. This is in keeping with the increment of the main transition temperature (T_m) of dimyristoylphosphocholine by betaine. More specifically, T_m was increased progressively by this solute, by 5 degrees at 5 M betaine (Söderlund et al., *unpublished results*). A similar increment is observed at a hydrostatic pressure of ~200 atm (Reyes Mateo et al., 1993).

As γ increases the number of interfacial water molecules is reduced due to augmented lipid lateral packing (i.e. decrease in the mean molecular area per lipid). Bagatolli et al. (1998) reported a linear correlation between intermolecular distances (ID) and GP. Comparison of the Laurdan GP value for POPC LUVs at 25 ^{o}C in the absence of kosmotropes to the data by Bagatolli et al. (1998) yields an intermolecular distance of ~2.5 Å. Following this line of analysis we may further estimate the decrease in ID due to increasing γ, which reveal a linear reciprocal correlation. **(Fig. 29)** This provides a method for estimating the effects of γ and Π on the lipid lateral packing in bilayers.

The effect of kosmotropes thus provides a powerful means for the cell to modulate the physical state and the funtion of membranes and activity of membrane proteins via changes in water structure. However, this effect would not be limited to integral membrane proteins only. The denaturation temperature of soluble proteins increases as γ is increased (Kaushik and Bhat, 1998). We have previously shown that betaine decrease the hydrodynamic volume and increase the molecular packing of a soluble

protein, *Humicola lanuginosa* lipase (Söderlund et al., 2002). The impact of the increase in γ by betaine on the protein-water interface is thus essentially analogous to the augmented packing and decreased hydration of the lipid bilayer reported here.

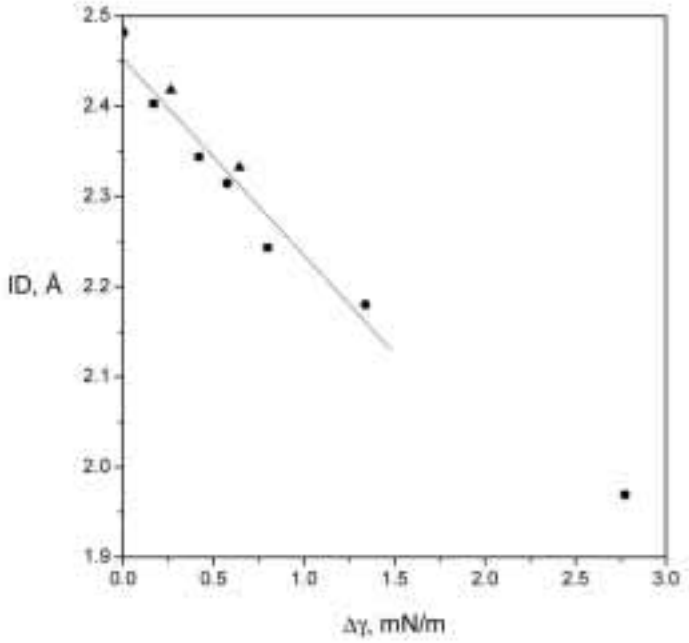


Figure 29. Intermolecular distance (ID) as a function of Δγ. Changes in γ are due to solutes.

Cells cannot control the chemical potential of pure water, but can change the water activity by accumulating different solutes (Rand et al., 2000). A change in water activity can have different responses on macromolecules. Isolated proteins undergo reversible transitions and get dehydrated, or kinetically find it more difficult to get to their more hydrated conformation (Parsegian and Rand, 1995). The osmotic work is observed as a change to a more dehydrated state (Parsegian and Rand, 1995). For single unilamellar liposome an analogous behaviour can be expected.

6. CONCLUSIONS

The stopped-flow fluorescence measurements demonstrated that the hydrophobic and electrostatic doxorubicin binding sites in liposomes can be distinguished by their different fluorescence decay halftimes. These different halftimes were utilized for studying membrane lateral organization on the course of main transition. From these measurements it can be concluded for the first time that binding of a drug to a membrane can be controlled by the formation of domains enriched in acidic phospholipids and with high local negative surface charge density.

The present results show that the interaction of CsA with membranes containing cholesterol are much more complex than those revealed in previous studies with pure PC bilayers. CsA was shown to change the thermal phase behaviour of the membrane, and also to alter the lateral organization in monolayers on a micrometer scale. This was the first time that a drug was directly observed to affect the lateral heterogeneity. There is a large body of evidence showing that cholesterol affects a number of processes of diverse nature in different cells. Moreover, organization of cholesterol in membranes can be anticipated to be critical to its functions. As the CsA-induced changes in membrane lateral heterogeneity were modulated by cholesterol, CsA can be expected to have different effect(s) on the membrane properties (and functions) in different cells and cell organelles.

All three neuroleptic drugs (CLZ, CPZ, and HPD) caused changes in the membrane lateral heterogeneity. The effects of these drugs were not identical, indicating the changes not to be caused by non-specific drug-lipid interactions. The atypical neuroleptic drug CLZ had different effects on the membrane lateral heterogeneity than the conventional neuroleptic drugs CPZ and HPD. Accordingly, the mode of membrane interaction of CLZ was less dependent on the electrostatic interactions than those of CPZ and HPD.

Definitive conclusions on the pharmacological significance of the findings on the effects of drugs on the lateral heterogeneity is premature at this stage. The drug induced effects are likely to be strongly dependent on multiple factors such as membrane lipid composition, pH, osmolarity, ionic strength and compound itself. Yet,

in conjunction with the importance of coupling between organization and function in biomembranes the present studies do indicate that efforts along these lines may provide novel insights to the understanding of the molecular mechanisms of action and/or adverse effects of membrane-associating drugs.

Studies with betaine, choline chloride, and sucrose revealed a correlation between surface tension and interfacial dynamics of lipid bilayers, as measured by Laurdan GP, whereas osmotic pressure had less effect on GP. These observations suggest a γ-mediated mechanism for controlling the functional and structural properties of lipid membranes.

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