LATERAL HETEROGENEITY IN MODEL MEMBRANES

- INDUCEMENTS AND EFFECTS -

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ABSTRACT

One of the key issues in modern biophysics is the connection between the structure, organization and function of biomolecules and their supramolecular assemblies. In the present thesis the organization of model lipid membranes is investigated by fluorescence spectroscopy, differential scanning calorimetry (DSC) and monolayer techniques.

The mechanism of the main phase transition and lateral heterogeneity of the lipid bilayer in the course of this process is studied. The coexistence of 'fluid' and 'gel' domains is evidenced by a transient increase in the efficiency of a pyrene labeled lipid derivative to form excimers in the vicinity of the phase transition temperature. Furthermore, results from resonance energy transfer (RET) between pyrene and three different acceptor fluorophores suggest the former to accumulate into the interfacial boundaries between the domains.

The rates for binding and dissociation of a peripheral membrane protein cytochrome *c* (cyt *c*) to vesicles is assessed by monitoring the decrease in pyrene monomer emission due to RET between pyrene lalbeled lipid PPDPG residing in the vesicles and the heme of cyt *c*. Both of these processes are controlled by the lipid composition and organization of the membrane, and slow down with the increasing contents of acidic phospholipid, suggesting a formation of cooperative hydrogen-bonded networks by deprotonated and protonated phosphatidylglycerols (PG).

The pharmaceuticals lidocaine, propranolol, and gentamycin bind avidly to phospholipid membranes and alter their structural dynamics as shown by excimer formation of PPDPG and fluorescenec anisotropy of DPH. Upon binding the cationic drugs induce deprotonation of PGs, and eventually dissociate cyt *c* from liposomes resulting in an increase in pyrene emission intensity. The present results are in accordance with multiple acidic phospholipid binding sites in cyt *c*.

The neuroleptic drugs clozapine (CLZ), chlorpromazine (CPZ), and haloperi-

dol (HPD) associate with lipid membranes changing the thermal behaviour of vesicles and domain morphology of monolayers as shown by DSC and fluorescence microscopy, respectively. By varying the lipid composition it is shown that in the membrane association the contribution of hydrophobic (vs. electrostatic) forces is more important for the atypical neuroleptic CLZ than for the conventional neuroleptics CPZ or HPD. These results support the view that membrane partitioning drugs could exert part of their effects by changing the lateral organization and thus also the functions of biomembranes.

CONTENTS

LIST OF ABBREVIATIONS

ACTH 1-24	adrenocorticotropin 1-24
bisPDPC	1,2-bis[(pyren-1-yl)decanoyl]-*sn*-glycero-3-phosphocholine
brainPS	brain phosphatidylserine
C	colocalization parameter
CLZ	clozapine
CPZ	chlorpromazine
cyt *c*	cytochrome *c*
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DPH	1,6-diphenyl-1,3,5-hexatriene
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPF	1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamino-N-(5-fluoresceinthiocarbamoyl)
EDTA	ethylenediaminetetraacetic acid
egg PC	egg phosphatidylcholine
egg PG	egg phosphatidylglycerol
F-H1	FITC labeled histone H1
FITC	fluorescein 5-isothiocyanate
GTM	gentamycin
$[GTM]_{50}$	gentamycin concentration producing 50 % reversal of fluorescence quenching
H1	histone H1
Hepes	N-(2-hydroxyethyl) piperazine-N'-2-ethanesulphonic acid
HPD	haloperidol
I_e/I_m	ratio of excimer and monomer fluorescence
K_{19}	polylysine
LDC	lidocaine
$[LDC]_{50}$	lidocaine concentration producing 50 % reversal of fluorescence quenching

LUV	large unilamellar vesicle
MLV	multilamellar vesicle
Myr-KRTLR	myristoylated Lys-Arg-Thr-Leu-Arg
NBD-chol	22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23, 24-bisnor-5-cholen-3β-ol
NBD-PC	1-palmitoyl-2-(N-4-nitrobenz-2-oxa-1,3-diazol) aminocaproyl-*sn*-glycero-3-phosphocholine
PG	phosphatidylglycerol
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol
PPDPC	1-palmitoyl-2[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine
PPDPG	1-palmitoyl-2[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphoglycerol
PRP	propranolol
$[PRP]_{50}$	propranolol concentration producing 50 % reversal of fluorescence quenching
PS	phosphatidylserine
RET	resonance energy transfer
RFI	relative fluorescence intensity
RFI_{max}	extent of maximal recovery of fluorescence
T_m	main transition temperature
T_p	pre transition temperature
T*	temperature of excimer formation maximum
X_z	mole fraction of compound z

LIST OF ORIGINAL CONTRIBUTIONS

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

I. Jutila, A. and Kinnunen, P.K.J. Novel features of the main transition of dimyristoylphosphocholine bilayers revealed by fluorescence spectroscopy.
Journal of Physical Chemistry B **101** (1997) 7635-7640.

II. Subramanian, M., Jutila, A., and Kinnunen, P.K.J. Binding and dissociation of cytochrome *c* to and from membranes containing acidic phospholipids.
Biochemistry **37** (1998) 1394-1402.

III. Jutila, A., Rytömaa, M., and Kinnunen, P.K.J. Detachment of cytochrome *c* by cationic drugs from membranes containing acidic phospholipids: comparison of lidocaine, propranolol, and gentamycin.
Molecular Pharmacology **54** (1998) 722-732.

IV. Jutila, A., Söderlund, T., Pakkanen, A.L., Huttunen, M., and Kinnunen, P.K.J. Comparison of the effects of clozapine, chlorpromazine, and haloperidol on membrane lateral heterogeneity.
Chemistry and Physics of Lipids (submitted).

INTRODUCTION

One of the great unraveled mysteries concerning the structure of biomembranes is the high diversity of different lipid species. In spite of the the extensive scientific effort in the field it is still largely unknown why the composition and organization vary so much from one lipid membrane to the next and what are the highly sensitive control mechanisms. The self-evident answer to the former question is the structural functionality, in other words the various functions of the biomembranes are controlled by the lipid composition. However, this oversimplified answer raises another even more intriguing questions: how do the molecular level changes in lipid composition or organization alter the macroscopic properties and furthermore the interactions of the complex supramolecular assembly?

The core of the thesis is the study concerning the still widely disputed mechanism of the main phase transition, and the organization of the phospholipid bilayer in the course of this process (I). Although cell membranes do not exhibit phase transitions *in vivo*, this kind of basic biophysical study of the organization of bilayers and the forces between its components utilizing structurally relatively simple model membranes is essential in order to understand the function of complex biomembranes.

In vivo, a vast legion of essential membrane proteins and enzymes are in close interaction with lipid bilayers, and their functions and activities can be expected to vary with the properties of the lipid phase. Accordingly, the basic physico-chemical angle taken in the first contribution is further widened in the accompanying studies, where kinetics of the interactions between a well characterized peripheral membrane protein cytochrome *c* (cyt *c*) and lipid bilayer are altered by varying the composition of the latter (II, III).

The conventional view in pharmacology is that the mechanism of action for most drugs is merely receptor mediated. However, a large number of pharmaceutical compounds are known to bind and penetrate into lipid bilayers,

and cause changes in their physico-chemical properties, such as lateral pressure or surface charge density. This may offer an alternative way of action as changes in the lipid environment of the receptors can be expected to change their functionality. The action of six different drugs, namely the b-adrenergic blocking agent propranolol, the local anesthetic lidocaine, the aminoglucosidic antibiotic gentamycin, and three neuroleptic drugs clozapine, chlorpromazine, and haloperidol, are addressed in the studies examining their effects on the membrane organization and dynamics, and for the three first mentioned the subsequent displacement of cytochrome *c* (III, IV).

Unfortunately studying structural dynamics of highly complex supramolecular assemblies, such as biomembranes, on a molecular level is unrealistic at present. The approach taken here is to mimic biomembranes by using liposomes or monolayers consisting of only few different lipids species. Fluorescence spectroscopy is an ideal method for studying this kind of systems, as only trace amounts of fluorophore-labeled lipids are required to be incorporated into membranes for efficient excimer formation or resonance energy transfer yielding information on the dynamic lateral organization of the membrane.

REVIEW OF THE LITERATURE

LATERAL HETEROGENEITY IN MEMBRANES

Lipid bilayer is the main structural feature in biomembranes providing the semipermeability essential for the function of these supramolecular assemblies. In addition to its conventional roles as a structural matrix for proteins and a diffusion barrier (Singer & Nicolson, 1972), the connection between the structure and function of biomembranes, i.e. functional ordering, has received increasing attention in recent years (Kinnunen, 1991; 2000). Lateral heterogeneity and packing defects may facilitate a number of biological functions of the membrane, and in this context it is relevant to understand the basic physical chemistry of lipids and the forces controlling their lateral ordering and diffusion. In brief, laterally separated phases within bilayer, i.e. domains, may be induced by temperature (Mouritsen, 1991), lipid-lipid (Lehtonen et al., 1996) or lipid-protein (Mouritsen & Bloom, 1984) hydrophobic mismatch, an enzymatic cleavage of lipids (Holopainen et al., 1998), surface electrostatic associations (Rytömaa & Kinnunen, 1996), or hydrogen bonding between lipid headgroups (Söderlund et al., 1999b).

Number of studies have indicated heterogeneous lipid organization to have several potential functions in biomembranes. Concerning the present thesis, binding of charged macromolecules, such as cyt *c* and H1, to lipid bilayers is controlled by the composition of the lipid domains (Kinnunen et al., 1994; Rytömaa & Kinnunen, 1996). Also, the interfacial regions between the domains are poorly ordered and contain structural defects that enhance the leakage through the membrane and easily accomodate 'impurities' such as drug molecules (Mouritsen & Jørgensen, 1998). At this point, it is relevant to mention that these interactions may induce alterations in the structural dynamics of both participants, as follows. The binding of a positively charged species to the membrane neutralizes negative surface charge density and subsequently increases

the deprotonation of acidic phospholipids causing lateral re-organization (Träuble, 1976). On the other hand, protein conformation is sensitive to its environment and can be expected to change upon association to membrane (Cortese et al., 1998; Tuominen et al., 2001). Furthermore, the lateral diffusion of membrane components provides a potential way to control the kinetics of enzymatic reactions taking place on the surface. An additional link between the lipid domain formation and biomembrane functions is indicated by the studies on the mechanical properties of bilayers, namely bending rigidity and lateral compressibility (Heimburg, 1998). Lateral heterogeneity softens the bilayer, making it amenable to a number of biological functions, including membrane fusion, vesiculation, and cytosis.

MAIN PHASE TRANSITION OF LIPID BILAYER

Thermally-induced transition of lipid bilayers from a relatively ordered crystalline-like gel state (L_β) existing at lower temperatures to a relatively disordered fluid-like state (L_α) at higher temperatures is driven by the entropy gain arising from acyl chain rotational isomerism. On a molecular level, the lipid hydrocarbon chains are converted from largely all-*trans* conformation in the gel state to a more orientationally disordered state characterized by the presence of a number of *gauche* conformations and greatly increased rate and extent of molecular motions. Thus, the melting of the hydrocarbon chains is accompanied by an increase also in the intermolecular entropy. On the other hand, the internal energy of the system is increased in the transition process as rotational isomerism decreases Van der Waals attractions between the hydrocarbon chains, and increases hydrophobic exposure due to lateral expansion (Bloom et al., 1991; Bagatolli & Gratton, 1999) of the membrane. Basically, the thermodynamics of the lipid main phase transition is determined by the balance of these opposing factors.

The process of the main phase transition provides an useful model for studying interactions in lipid vesicles on the molecular level. Several macroscopic physical properties of membranes are known to exhibit anomalies in the vicinity or at the temperature of the main phase transition (Table 1). Several of

TABLE 1. *Macroscopic physical properties of membranes exhibiting anomalies in the temperature region of the main phase transition.*

MEMBRANE PROPERTY	REFERENCE
heat capacity	1
lateral fluctuations	2
lateral diffusion	3
relaxation time	4-9
bending elasticity	10-12
transversal compressibility	13
lateral compressibility	14-16
permeability	17-20
drug release	21,22
thickness	23,24
area	2,25
domain boundary length	26
vesicle shape	25
activity of phospholipase C	27,28
activity of phospholipase A_2	29,30

References: 1 Mabrey & Sturtevant, 1976; 2 Bloom et al., 1991; 3 Vaz et al., 1989; 4 Kanehisa & Tsong, 1978; 5 Gruenewald et al., 1981; 6 van Osdol et al., 1991; 7 Mitaku et al., 1983; 8 Harkness & White, 1979; 9 Jørgensen et al., 1996; 10 Hønger et al., 1994; 11 Meleard et al., 1997; 12 Fernandez-Puente et al., 1994; 13 Alakoskela & Kinnunen, 2001; 14 Nagle & Scott, 1978; 15 Evans & Kwok, 1982; 16 Needham & Evans, 1988; 17 Papahadjopoulos et al., 1973; 18 Nagle & Scott, 1978; 19 Maynard et al., 1985; 20 Mouritsen et al., 1995; 21 Gerasimov et al., 1996; 22 Anyarambhatla & Needham, 1999; 23 Wilkinson & Nagle, 1981; 24 Lemmich et al., 1995; 25 Bagatolli & Gratton, 1999; 26 Freire & Biltonen, 1978; 27 Gabriel et al., 1987; 28 Thuren & Kinnunen, 1991; 29 Op Den Kamp et al., 1975; 20 Menashe et al., 1986.

these features have been suggested to be connected to the presence of two co-existing phases in the transition region, i.e. the presence of fluid and gel micro-domains and their interfacial boundary (Doniach, 1978; Marsh et al., 1977; Freire & Biltonen, 1978; Mouritsen et al., 1995; Bagatolli & Gratton, 1999). Infrared

(Mellier et al., 1993) and microwave (Enders & Nimtz, 1984) studies have suggested the main transition to be a two step process, where changes in the headgroup conformation would precede chain melting of the lipids. In biomembranes the lateral diffusions of membrane-bound particles and transport of small molecules, such as drugs, can be driven by the lateral motion of the lipids (Galla et al., 1979). Furthermore, unraveling the mechanism of the phase transition of lipid bilayer is critical for novel applications, such as liposomal drug delivery (Mouritsen & Jørgensen, 1998; Anyarambhatla & Needham, 1999).

Fluorescence spectroscopy of membrane embedded pyrene derivatives is a powerful tool for studies on structural dynamics of supramolecular assemblies. Pyrene-labeled lipids, such as 1-palmitoyl-2[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphatidylcholine (PPDPC), included into model membranes form excimers in a concentration dependent manner (Kinnunen et al., 1993; Lianos & Duportail, 1992). In samples exhibiting lateral heterogeneity, e.g. a vesicle in the phase transition region, efficiency of excimer formation is controlled by the rate of lateral diffusion and lateral enrichment, i.e. local concentration of the probe, or both. Furthermore, there is strong experimental and theoretical evidence for PPDPC to distribute as hexagonal superlattice at certain critical concentrations and conditions (Somerharju et al., 1985; Tang & Chong, 1992; Sugar et al., 1994; Chong et al., 1994). Locally this might be the case also for a bilayer in the course of the main transition.

ASSOCIATION OF CYT C WITH LIPID BILAYERS

The lipid environment of bilayer provides not only a structural support for integral membrane proteins. Accordingly, changes in the organization and dynamics of the membrane can lead to alterations in the functions of membrane proteins as shown for P-glycoprotein (Romsicki & Sharom, 1999) and the opioid receptor (Lazar & Medzihradsky, 1992), for example. The ligand affinity of the latter has been shown to be sensitive to the changes in the 'fluidity' in the interfacial region, but insensitive to changes in the hydrocarbon core (Lazar & Medzihradsky, 1992). Lipid peroxidation has been shown to modulate the function of 5-hydoxytryptamine receptor by altering the physical properties of the

lipid membrane (Rego & Oliviera, 1995). A model involving coupling of the membrane lateral pressure profile to the conformation and function of integral membrane proteins has been recently forwarded by Cantor (1997) and could provide a mechanistic basis for the effects of membrane composition on opioid receptor function, for instance. While the above findings demonstrate the importance of the lipid environment to the function of proteins, they also reveal the importance of drug-lipid interactions leading to changes in membrane organization, dynamics and function, and further suggest that these properties could be considered as potential drug targets.

Cytochrome *c* (cyt *c*) is a roughly globular 13 kD protein that consists of 104 amino acid residues and at neutral pH carries a positive net charge of +8. It functions in the mitochondrial respiratory chain between ubiquinone:cytochrome *c* reductase and cytochrome *c* oxidase with its heme moiety switching between ferro and ferri forms. The mode of interaction between cyt *c* and the membrane is coupled to the lipid composition and organization of the latter, and two different binding sites, nominated as A- and C-site, in cyt *c* have been postulated. (Rytömaa et al., 1992; Rytömaa & Kinnunen, 1994;1995). The former electrostatic interaction dominates at low contents of acidic phospholipid, such as phosphoglycerol (i.e. glycerol-3-phosphate), in the membrane and is reversed by ATP. This nucleotide competes with the deprotonated acidic phospholipid for the same binding site in cyt *c* and induces conformational changes in the protein (Rytömaa & Kinnunen, 1994; Feng & Englander, 1990; Tuominen et al., 2001). These effects of ATP are highly pH dependent with decreasing efficiency under more acidic conditions. On the contrary, the hydrogen bonding via C-site predominates at high contents of the acidic phospholipid and is insensitive to ATP (Rytömaa et al., 1992; Rytömaa & Kinnunen, 1994). In addition, both modes of binding have been postulated to involve hydrophobic interaction between the protein and the acidic phospholipid. One plausible mechanism is the so-called extended lipid anchorage, where the acyl chains of a phospholipid are pointing to opposite directions, i.e. one of the chains penetrates into the hydrophobic cavity within cyt *c*, while the other resides in the bilayer (Rytömaa & Kinnunen, 1995). The described model is in accordance with a recent study, where membrane bound cyt *c* adapted two different conformations with different electron transfer activities depend-

ing on the ionic strength (Cortese et al., 1998) suggesting a novel mechanism for cyt *c*-mediated electron transfer regulation. Similar ideas were presented in a study where binding of cyt *c* to acidic vesicles is shown to be driven by electrostatic interactions, wherafter the local low pH at the membrane surface induces changes in its tertiary structure (Pinheiro et al., 1997). Along the same line, the disruption of Met 80 coordination to the heme iron leading to conformational changes in cyt *c* upon binding has been shown (Heimburg et al., 1991; Spooner & Watts, 1991a,b, 1992; Pinheiro & Watts, 1994a,b).

Recently, cyt *c* has been shown to play a central role in programmed cell death (Yang et al., 1997; Kluck et al., 1997). Accordingly, the release of cyt *c* from mitochondria into the cytoplasm is the rate limiting step in the entry of a cell into the apoptosis. This is in accordance with recent work by Jemmerson et al. (1999) demonstrating similar changes in the conformation of cyt c in apoptosis and lipid association. This would also provide a physiological rationale for the multiple phospholipid binding sites in cyt *c*.

Histone H1 (H1) is a basic protein that plays a major role in chromatin condensation and regulation of gene expression in the cell nucleus. The affinity of H1 to membranes containing acidic phospholipids exceeds that of cyt *c* as evidenced by the efficient displacement of cyt *c* from vesicles by H1 (Rytömaa & Kinnunen, 1996). In the same study dissociation of cyt *c* from membranes was shown to be induced also by polycationic model peptides polylysine K_{19}, myristoylated peptide myr-KRTLR, and fragment of adrenocorticotropin hormone ACTH 1-24.

EFFECTS OF DRUGS ON MEMBRANES

Understanding of drug-lipid interactions is important for a number of reasons. First, lipid membranes provide the major barrier against the passive diffusion of drugs into the intestinal cells and into specific tissues, such as the blood-brain barrier. Elucidation of the mechanisms affecting the passive diffusion of compounds through the lipid bilayer are thus of primary importance in drug development. Second, the overexpression of the P-glycoprotein is one of the major causes for multidrug resistance in human cancers (Romsicki & Sharom,

1999). These transporters are integral membrane proteins, and the interaction between the protein and ligand requires the latter to be located in the membrane. Third, binding of a drug to lipids can lead to alterations in the function of membrane proteins. This could be involved in the actual mechanism of action or in the adverse effects of drugs, as proposed for the lung toxicity of amiodarone (see review by Reasor & Kacew, 1996) and the cardiotoxicity of doxorubicin (Goormaghtigh et al., 1982). Fourth, understanding of drug-lipid interactions is crucial in the design of liposomes for use as drug carriers. Finally, the lipid membrane could represent the actual target for the drug (Kinnunen, 1991; Söderlund et al., 1999a), as shown for amphotericin B (Bolard, 1986), and antimicrobial peptides (Bechinger, 1997).

One of the aims of the thesis was to study the effects of six cationic drugs, namely local anesthetic lidocaine (LDC), b-adrenergic blocking agent propranolol (PRP), aminoglucosidic antibiotic gentamycin (GTM), and three neuroleptics clozapine (CLZ), chlorpromazine (CPZ), and haloperidol (HPD), on the organization and dynamics of lipid bilayers, and furthermore for the three first mentioned drugs compare their efficiencies in displacing cyt *c* from liposomes containing acidic phospholipids (Fig. 1). As all these drugs possess net positive charge(s) they can be expected to bind avidly to membranes containing acidic phospholipids, and subsequently decrease the negative surface charge density (Roucou et al., 1995), which in turn can be expected to increase the deprotonation of the acidic phospholipids (Träuble, 1976). As explained in the previous chapter, at $X_{PG} = 1.00$ this would change the acidic phospholipid binding site in cyt *c* from C to A.

Lidocaine (Hanpft & Mohr, 1985; Schlieper & Steiner, 1983; Davio & Low, 1981; Ueda et al., 1994), propranolol (Kubo et al., 1986; Hanpft & Mohr, 1985; Schlieper & Steiner, 1983; Albertini et al., 1990), and gentamycin (Brasseur et al., 1984; Chung et al., 1985; Kubo et al., 1986; Gurnani et al., 1995) have been previously shown to bind avidly to lipid bilayers yet the pharmacological significance of these interactions remains uncertain. Both LDC and PRP are amphiphilic and partially penetrate into the hydrophobic core of the membrane. The latter compound has been suggested to have two different binding sites in phospholipid membranes, as follows (Kodavanti & Mehendele, 1990; Kubo et al., 1986). The high-affinity, low-capacity binding site is probably in

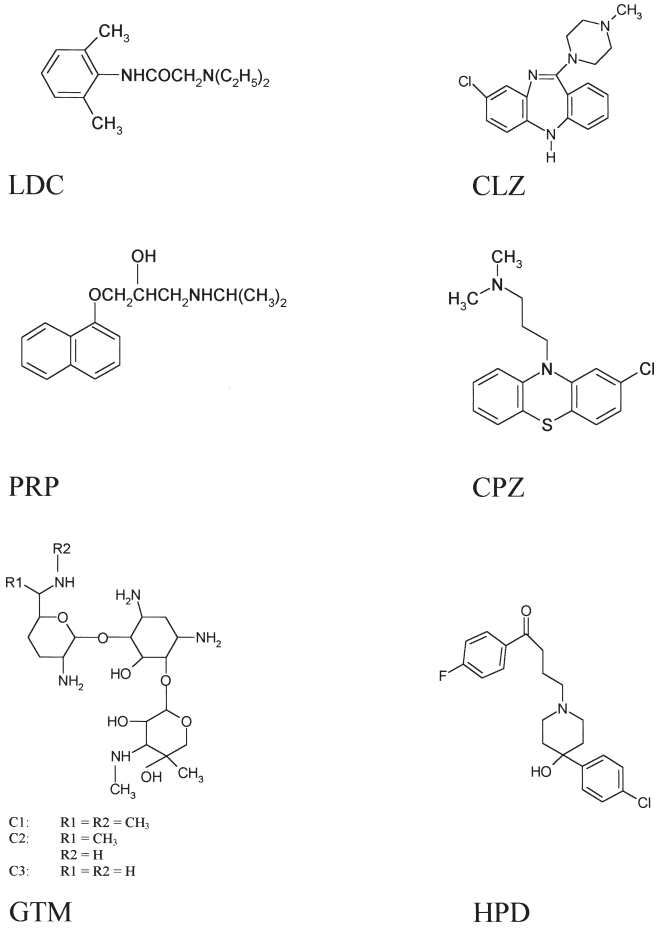

FIGURE 1*. Molecular structures of the studied drugs lidocaine (LDC), propranolol (PRP), gentamycin (GTM), clozapine (CLZ), chlorpromazine (CPZ), and haloperidol (HPD).*

the surface and involves primarily electrostatic forces, whereas the low-affinity, high-capacity site has been proposed to reside in the interior of the lipid bilayer and is mainly due to the hydrophobicity of the drug. Using X-ray diffraction Albertini et al. (1990) found PRP to increase water layer thickness on DPPC membrane surface. Compared to PRP the affinity of LDC to membranes is

less and its effects on properties of bilayers, such as zeta potential or phase transition temperature, are not as pronounced (Hanpft & Mohr, 1985; Schlieper & Steiner, 1983). Ueda et al. (1994) demonstrated by IR-spectroscopy LDC to release hydrogen-bonded water from the phosphate and glycerol moieties of DPPC. GTM is hydrophilic and its binding to liposomes requires the presence of acidic phospholipids (Brasseur et al., 1984; Chung et al., 1985; Kubo et al., 1986). The electrostatic association of GTM to liposomes results in charge neutralization and tightening of lipid packing (Gurnani et al., 1995). Due to its net positive charge (~+3) GTM molecules should be able to complex with three negatively charged phospholipids. Minor hydrophobic interaction between GTM and membranes is indicated by the penetration of the drug into phospholipid monolayers (Brasseur et al., 1984).

CPZ has been reported to associate with the headgroup region of lipid bilayer forming a 1:1 complex with acidic phospholipid (Stuhne-Sekalec et al., 1987), and also to penetrate into the acyl chain region (Römer & Bickel, 1979). Depending on membrane lipid composition and phase state both an increase as well a decrease in the acyl chain order in membranes have been reported to be caused by CPZ (Neal et al., 1976; Römer & Bickel, 1979). 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 & Oliviera, 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-adenergic, and dopamine receptor ligands (Rego & Oliviera, 1995).

Considering lipid membranes as potential drug targets may be particularly relevant when considering neuroleptics. The reconstituted dopamine D_2-receptor requires a lipid mixture of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine 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 cyt *c* oxidase by doxorubicin (Goormaghtigh et al., 1982). CPZ causes alterations in the phospholipid compositions of different cellular membranes (Stuhne-Sekalec et al., 1987; Singh et al., 1992). The increase in the content of acidic phospholipid and increased unsaturated/saturated lipid ratio in particular could represent adaptive responses (Stuhne-Sekalec et al., 1987). Interestingly, changes in the cell membrane phospholipid compositions in the brain of schizophrenic patients have been related to the onset of clinical symptoms (Pettegrew & Minshew, 1992). Recently, ω-3 fatty acid supplemented diet was shown to improve the course of illness in bipolar disorder, and altered membrane properties and an effect of this modulation on signal transduction were suggested as the mechanism of action (Stoll et al., 1999).

AIMS OF THE PRESENT STUDY

The overall theme of this thesis is lateral organization of lipid membranes. The aims were:

(i) to gain insight on the mechanism of the main phase transition of lipid bilayer on molecular level,

(ii) to determine the effects of lidocaine, propranolol, gentamycin, clozapine, chlorpromazine, and haloperidol on the structural dynamics of phospholipid membranes, which could represent a possible alternative mechanism of pharmacological action for these drugs,

(iii) to characterize the role of lipid composition in the binding and dissociation rates of liposomes and cytochrome *c*,

(iv) to resolve how drugs, namely lidocaine, propranolol, and gentamycin, interfere with the interaction between cytochrome *c* and the membrane,

(v) to compare the effects of atypical neuroleptic clozapine to those of conventional neuroleptics chlorpromazine and haloperidol on the organization of the lipid membranes.

MATERIALS AND METHODS

MATERIALS

Hepes, EDTA, horse heart cyt *c* (type VI, oxidized form), K_{19}, egg PC, egg PG, cholesterol, FITC, and all the studied drugs (LDC, PRP, GTM, CLZ, CPZ, HPD) were purchased from Sigma. The pyrene labeled phospholipid derivatives PPDPC and PPDPG were from K&V Bioware (Espoo, Finland). POPG, POPC and NBD-PC were obtained from Avanti Polar Lipids (Alabaster, AL, USA), DPPF and NBD-chol from Molecular Probes (Eugene, OR, USA), DPPC from Coatsome (Amagasaki, Hyogo, Japan), and DMPC from Princeton Lipids (Princeton, NJ, USA). Synthetic ACTH 1-24 was a gift from Ciba-Geigy AG (Basel, Switzerland). Myr-KRTLR was from Bachem (Bubendorf, Switzerland) and Na_2-salt of ATP was purchased from Boehringer Mannheim (Germany). DPH was purchased from EGA Chemie (Steinheim, Germany). Histone H1 had been purified from calf thymus (Johns, 1976) and labeled with FITC according to the method of Favazza et al. (1990). The buffer used in all experiments was 20 mM Hepes, 0.1 mM EDTA, pH 7.0 prepared of water freshly deionized in a Milli RO/Milli Q (Millipore) filtering system.

LIPOSOME PREPARATION

The appropriate amounts of lipids and drugs were first mixed in chloroform, whereafter the solvent was removed under a stream of nitrogen. The residue was kept under reduced pressure for two hours and then hydrated in the buffer to obtain multilamellar vesicles (MLV), which were used as such in the DSC experiments (IV). To prepare large unilamellar vesicles (LUV) the hydrated lipid mixtures were extruded through two polycarbonate filters (100 nm pore size, Nucleopore, Pleasanton, CA, USA) with a LiposoFast homogenizer (Avestin,

Ottawa, Canada). In the experiments requiring fluorescence labeling of vesicles the utilized X_{probe} was varied between 0.002 and 0.016. These contents of the fluorescent probes yield well resolved emission signals while minimal perturbation of the packing of the unsaturated matrix lipids and negligible inner filter effect can be expected.

STEADY STATE FLUORESCENCE SPECTROSCOPY

Steady-state fluorescence measurements were carried out with a Perkin Elmer LS 50B Luminescence Spectrometer. The instrument is equipped with magnetic stirrer and circulating waterbath to maintain constant temperature (25 °C, unless otherwise indicated). The pyrene labeled lipids PPDPC, PPDPG, and bisPDPC were excited at 344 nm, and monomer and excimer emission was detected at 398 and 480 nm, respectively. When indicated, two fluorescent probes were simultaneously present in the LUVs (I). More specifically, PPDPC was used as a donor in resonance energy transfer, while either NBD-chol, NBD-PC, or DPPF were employed as acceptors. Lipid binding and detachment of cyt *c* was assessed (Mustonen et al., 1987; Rytömaa et al., 1992; Rytömaa & Kinnunen, 1994; 1995) by monitoring the decrease in pyrene monomer emission due to resonance energy transfer between PPDPG and the heme of cyt *c* (II, III). The advantages as well as limitations of the use of pyrene-labeled lipids in energy transfer measurements have been discussed elsewhere (Mustonen & Kinnunen, 1993; Kaihovaara et al., 1991; Kinnunen et al, 1993). In addition to the measurements employing pyrene excimer formation, the effects of drugs (LDC, PRP, and GTM) on the membrane dynamics was studied utilizing fluorescence anisotropy r for DPH embedded in lipid bilayer. For DPH 350 nm was used for excitation, and the horizontally and vertically polarized components of the emission were monitoredat 450 nm, and the anisotropy was calculated by the equation

$$r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ stand for intensities of parallel and antiparallel components of the emission, respectively (Lakowicz, 1999).

STOPPED-FLOW FLUORESCENCE SPECTROSCOPY

The rates for liposome association and detachment of cyt *c* were measured using Olis RSM 1000F stopped-flow spectrofluorometer (On-line Instrument Systems Inc., Bogart, GA, USA). Excitation for pyrene at 344 nm was provided by a water-cooled 450 W Xe arc lamp. Jets of reactants were injected from pneumatic syringes into the rapid mixing chamber with a deadtime of ≤ 2 ms, connected to the fluorescence observation chamber, and the emission spectra from 364 to 516 nm were recorded as a function of time. In order to assess the membrane association of H1 this protein was labeled with FITC while bisPDPC was included in liposomes as a fluorescence donor (Kõiv et al., 1995). Analogously to the studies on cyt *c*, this energy transfer couple utilizes the overlap between pyrene excimer emission and fluorescein absorption spectra. The kinetic data were fitted using either one- or two-exponential equation solved with nonlinear least-squares fitting procedures provided by the instrument manufacturer.

DIFFERENTIAL SCANNING CALORIMETRY

After equilibrating the MLVs on an ice-water-bath for at least 10 hrs, the endotherms were recorded using VP-DSC microcalorimeter (Microcal Inc., Northampton, MA,USA). Heating rate was 30 degrees/h and the final lipid concentration in the DSC cell was 0.4 mM. All scans were repeated to assure their reproducibility. The endotherms were analyzed using the routines of the software provided by Microcal.

COMPRESSION ISOTHERMS OF MONOLAYERS

Compression isotherms were recorded using μTrough S monolayer through (Kibron Inc., Helsinki, Finland) equipped with KBN129 high precision microbalance (Kibron Inc.) and a metal alloy probe to monitor surface pressure (π). The mixtures of lipids and neuroleptic drugs were dissolved in a mixture of hexane/isopropanol/water (70/30/2.5, by vol.), and spread on the air-buffer interface. After 5 min equilibration the film compression was started at constant rate of one Å^2/acyl chain/min using two symmetrically moving barriers. The compression isotherms are represented as π **vs** Å^2/acyl chain, where a drug molecule is taken as equivalent to one acyl chain. All monolayer measurements were done at ambient temperature (~+22-23 °C). The mean molecular areas occupied by the drugs in the film at any given surface pressure were calculated using the following equation:

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

where A_D is the surface area of the drug, 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, and X_D its mole fraction in the film. The partitioning of the drugs to the subphase was assumed to be neglible.

DRUG PENETRATION INTO MONOLAYERS

Penetration of CLZ, CPZ, and HPD into monomolecular lipid films was measured using magnetically stirred circular wells with a surface area of ~1.6 cm^2 and a subphase volume of 300 μl (Multiwell plate, Kibron Inc.). Surface pressure was monitored as descibed above. The indicated lipids were mixed in chloroform (~0.5 mg/ml) and spread on the air-water interface with a microsyringe. The monolayers were allowed to equilibrate for approx. 5 min to reach the indicated initial surface pressure values (π_0). The drugs (2 μl of 200 μM drug in DMSO) were injected into subphase to yield a final drug concentration of 1.3 μM. This amount of DMSO as such had no effect on the surface pressure.

After the increase in the surface pressure was complete, the difference between π_0 and the final surface pressure was taken as the increase in surface pressure ($\Delta\pi$). The data are represented as $\Delta\pi$ **vs** π_0, thus revealing the effect of increasing lateral packing density on the penetration of drug into monolayer (Brockman, 1999).

FLUORESCENCE MICROSCOPY OF MONOLAYERS

Compression isotherms for fluorescence microscopy were recorded as described above, with slight modifications. After the target pressure was reached the monolayer was allowed to stabilize for 10 min before the image was recorded through a quartz window on the bottom of the Langmuir through with a Zeiss IM-35 inverted fluorescence microscope (Jena, Germany) equipped with Nikon ELWD (20x) objective. 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, and it is to be emphasized that the images obtained are unlikely to represent true equilibrium. Yet, the results should be amenable to comparison as the equilibration times and compression rates were kept identical, and the observed domain morphologies were reproducible. In these experiments the subphase volume was 22 ml and the total amount of lipids and the drugs in the monolayer was 15 nmol. The molar ratio ($X = 0.05$) of the drug contained in the film would thus correspond to a subphase concentration of approx. 34 nM.

To emphasize the potential pharmacological relevance of the monolayer experiments, we want to point out that the concentrations of the drugs ($\approx$ 34 nM) required to induce the described effects on the domain morphologies are within the range of their therapeutic plasma concentrations (Dollery, 1991; Spina et al., 2000). Likewise, while varying for different receptors and their subtypes the dissociation constants for these neuroleptic drugs are in the range of 0.1 - 10 μM (Brody et al., 1998).

RESULTS

LATERAL HETEROGENEITY IN THE COURSE OF THE MAIN PHASE TRANSITION

The first motivating finding in the phase transition study was the transient peak in I_e/I_m **vs** temperature for PPDPC labeled LUVs (Fig. 2A). This peak (denoted by T*) does not shift upon three-fold increase in X_{PPDPC} (data not shown). Importantly, T* precedes the specific heat peak at T_m (23.9 °C), determined for these LUVs by DSC. The first derivative of this curve reveals a "baseline process" which progressively enhances I_e/I_m, while there is a transient increase beginning about four degrees below T_m (denoted by T_0) and reaching a maximum about two degrees higher (Fig. 2B). At T_m the I_e/I_m values return close to the ascending baseline attributed to the thermally enhanced excimer formation. Yet, $d(I_e/I_m)$ **vs** $(T - T_m)$ also shows a weaker process when $T > T_m$, which is

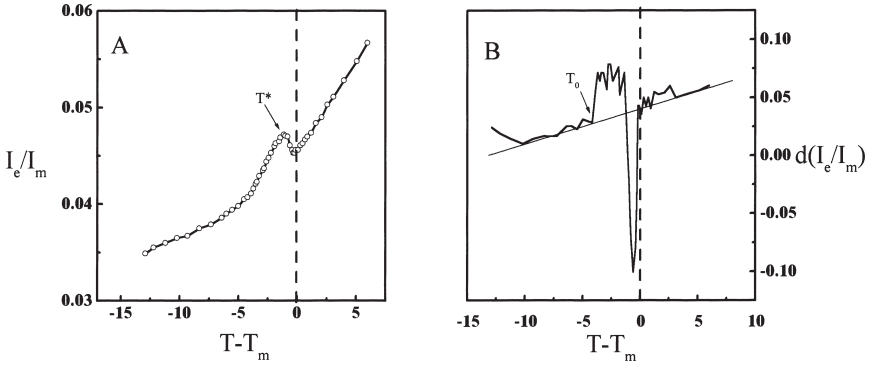

FIGURE 2. *Panel A: Excimer formation efficiency of PPDPC residing in large unilamellar vesicles (LUV) of DMPC. Main phase transition temperature T_m as indicated by differential scanning calorimetry (23.9 °C) is marked with the dashed line. Panel B: First derivative of the curve in panel A (see text for details).*

complete at ~2 °C above T_m. The local maxima in I_e/I_m could be due to lateral enrichment of the probe or enhanced lateral diffusion. As "fluidity" and lateral diffusion are gradually augmented upon the phase transition (Lange, 1986), lateral enrichment seems more plausible.

Upon approaching T_m formation of 'fluid' domains should ensue within the gel bulk of lipid bilayer (Doniach, 1978; Marsh et al., 1977; Freire & Biltonen, 1978; Mouritsen et al., 1995). To resolve between the enrichment of PPDPC into the (i) gel phase, (ii) fluid domains, or (iii) domain interface additional fluorescent lipids were incorporated into vesicles to act as resonance energy transfer (RET) acceptors for PPDPC excimer emission. The selected acceptors were NBD-chol (Mouritsen et al., 1995; Weis & McConnell, 1985; Hwang et al., 1995), DPPF (Lehtonen et al., 1996; Kõiv et al., 1995), and NBD-PC (Weis & McConnell, 1985), which are known to favor the interfacial, gel, and fluid environment, respectively. Colocalization parameter C was then defined as:

$$C = (I_0\text{-}I)/I_0$$

where I_0 and I are excimer emission intensities measured for PPDPC in the absence and presence of the acceptor, respectively. Accordingly, maximum ($I \rightarrow 0$) for C indicates augmented colocalization of the probes whereas minimum ($I \rightarrow I_0$) reports the probes being dispersed in the membrane.

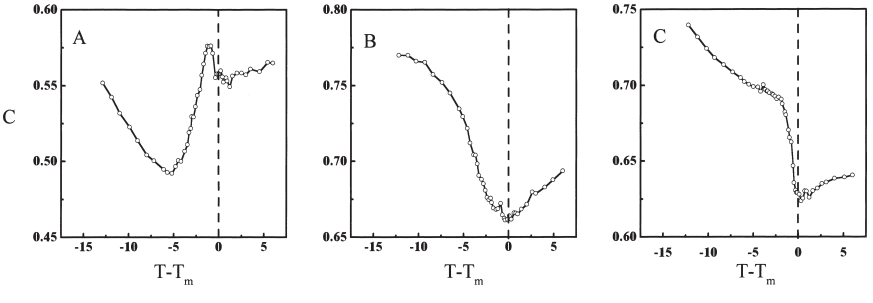

FIGURE 3. *Colocalization of PPDPC with NBD-chol (Panel A), DPPF (B), and NBD-PC (C).*

For PPDPC and NBD-chol, C **vs** ($T - T_m$) reveals colocalization in the gel phase to diminish with increasing temperature, and the first minimum is reached ~5 degrees below T_m (Fig. 3A). Thereafter a peak in colocalization is evident at T*, subsequently followed by a minimum at ~ T_m. Upon further increase in temperature above T_m, there is a slight increase in C. Assuming NBD-chol to reduce line tension between coexisting solid and fluid domains and to favor partitioning into the gel-fluid interface similarly to cholesterol (Mouritsen et al., 1995; Weis & McConnell, 1985; Hwang et al., 1995), our data strongly suggest the peak in I_e/I_m for PPDPC at T* to result from preference of this probe for the domain boundary. This would be in accordance with studies on the lateral distribution of pyrene in DODAC membranes (Pansu et al., 1993).

In the case of DPPF colocalization decreases when $T \rightarrow T^*$, whereafter a minimum follows at T_m (Fig. 3B). Above T_m colocalization of the chromophores is augmented. These data suggest colocalization of DPPF and PPDPC to decrease when the latter becomes enriched into the fluid-gel interface while DPPF remains in the gel domains.

For NBD-PC the colocalization curve is an intermediate between those recorded for NBD-chol and DPPF (Fig. 3C). The slow decrease in colocalization upon approaching T* as well as its more abrupt decline slightly below T_m is compatible with dissolving of microscopic domains enriched in both probes. The most likely reason for the formation of such microdomains well below T_m is minimizing free energy by reduction in the extent of perturbation of the packing of the gel state DMPC matrix. Following a minimum in C at T_m, there is a slight increase in RET between the two probes, coinciding with the temperature range of the post-transition process (Fig. 2B).

LIPID COMPOSITION CONTROLS THE KINETICS OF PROTEIN ASSOCIATION

BINDING OF CYT C TO LIPOSOMES

To study the attachment of cyt *c* to LUVs by the different postulated lipid binding sites (A-site and C-site) of cyt *c* the experiments were conducted at $X_{PG} =$ 0.20, 0.30, 0.40, 0.50, 0.60, 0.75 and 1.00. The time range for these processes

TABLE 2. *Halftimes (msec) for binding of cyt c (in the absence and presence of 5 mM ATP) and H1 to eggPG/eggPC LUVs, and the subsequent dissociation of the former by various agents. The concentrations used were those resulting in a saturating response in steady state fluorescence measurements.*

BINDING:				
	$X_{PG} = 0.20$		$X_{PG} = 1.00$	
	t_1	t_2	t_1	t_2
cyt *c*	4.7		6.2	46
cyt *c* + ATP	–b		16	
H1	7.9	50	52	185
DISSOCIATION:				
	$X_{PG} = 0.20$		$X_{PG} = 1.00$	
	t_1	t_2	t_1	t_2
ATP	5.9	39	–b	
NaCl	4.4	48	11.4	152
H1	17	145	204	1610
K_{19}	3.8	42	10	2500
myr-KRTLR	22	203	–b	
ACTH 1-24	5.7	63	250	6660

b) insignificant binding or dissociation

is 10^{-2} sec, and the half times at the lowest and highest acidic phospholipid content used are compiled in Table 2. In keeping with previous steady state fluorescence data the association of cyt *c* with eggPG/eggPC liposomes (25 μM) was fully saturated in the presence of 1 μM protein with little difference in fluorescence intensity in the studied X_{PG} range (Rytömaa & Kinnunen, 1994). Under these conditions the A-site mediated attachment of cyt *c* to acidic phospholipid at $X_{PG} = 0.20$-0.40 is evident as a single-exponential decay of pyrene monomer emission with the halftime for this process increasing with X_{PG}. Notably, at $X_{PG} \geq 0.50$ double-exponential fittings were required for satisfactory fits, while both of these two processes slowed down gradually upon further increase in X_{PG}.

In order to study the membrane association of cyt *c* selectively by the C-site the measurements were conducted in the presence of 5 mM ATP while X_{PG} was varied between 0.50 and 1.00 (Rytömaa & Kinnunen, 1994). These data were best fitted as a single-exponential decay with halftime increasing with X_{PG}. This blocking of A-site of cyt *c* by ATP provides further support to the notion that the faster component measured in the absence of ATP arises from an electrostatic interaction between cyt *c* and deprotonated PG.

BINDING OF H1 TO LIPOSOMES

Decrease in the fluorescence of bisPDPC labeled LUVs upon membrane association of FITC-H1 was two-exponential over the range of X_{PG} studied, and in resemblance to cyt *c* the halftimes were progressively prolonged upon increasing X_{PG} from 0.20 to 1.00. Association of H1 with liposomes containing PG attenuates lipid lateral diffusion and increases lipid acyl chain order as revealed by the decrease in I_e/I_m values for PPDPG as well by increase in fluorescence anisotropy of DPH (Rytömaa & Kinnunen, 1996). The H1 binding site has been estimated earlier to be constituted by approx. 20 phospholipids, and we measured the rate of the formation of this domain by monitoring I_e/I_m **vs** time at $X_{PG} = 1.00$. This process was single-exponential with a halftime of 59 msec, a slightly slower than the fast component of the membrane binding of FITC-H1 (52 msec). The difference of approx. 7 msec is likely to represent the time required for the simultaneous scavenging of the acidic phospholipids into the membrane domain underneath H1 and forming the protein binding site in the membrane.

DISSOCIATION OF CYT C FROM LIPOSOMES

High affinity binding sites for ATP have been described in cyt *c* (Corthésy & Wallace, 1986). At $X_{PG} = 0.20$ the A-site mediated binding of cyt *c* to deprotonated acidic phospholipids is prevented by ATP, as they compete for the same cationic binding site(s) in cyt *c* (Rytömaa & Kinnunen, 1994; Tuominen et al., 1997). After obtaining the rates for A- and C-site binding of cyt *c*, it was of high interest to study the release of this protein by ATP varying X_{PG} of LUVs

from 0.20 to 1.00. At $X_{PG} = 0.20$ the increase in pyrene fluorescence was two-exponential with halftimes of 5.9 and 39 msec (Table 2). At $X_{PG} = 0.30$ and 0.40 this process was one-exponential, and decelerated with increasing X_{PG}. Instead of releasing cyt *c* further quenching of fluorescence with complex kinetics was evident when ATP was added at X_{PG} in the range of 0.50 to 1.00, in keeping with ATP-induced conformational changes in membrane-bound cyt *c* resulting in more efficient RET (Rytömaa et al., 1992).

The electrostatic association of cyt *c* with acidic phospholipids is sensitive to ionic strength and increasing [NaCl] both dissociates cyt *c* from membranes as well as decreases the pK_a for the acidic phospholipid (Nicholls, 1974; Rytömaa et al., 1992; Träuble, 1976). When the content of the acidic phospholipid PG in vesicles increases progressively higher salt concentrations are required to detach cyt *c* from their surface (Rytömaa & Kinnunen, 1994). The dissociation of cyt *c* from LUVs by NaCl resulted in a double-exponential increase in fluorescence at all studied contents of acidic phospholipid. Similarly to the binding of the protein, also the dissociation by NaCl attenuated gradually with increasing X_{PG}.

It has been shown earlier that H1, and the cationic model peptides K_{19}, myr-KRTLR, and ACTH 1-24 are able to reverse the membrane binding of cyt *c* (Rytömaa & Kinnunen, 1996). The dissociation of cyt *c* by H1, K_{19}, and ACTH 1-24 from liposomes was double-exponential over the range of X_{PG} studied, from 0.20 to 1.00. Increasing affinity of cyt *c* to the vesicles was observed upon increasing X_{PG}, and myr-KRTLR was able to dissociate cyt *c* from membranes only at $X_{PG} < 0.50$. However, for all these polypeptides the halftimes for both components prolonged upon increasing X_{PG}.

LDC, PRP AND GTM ALTER THE STRUCTURAL DYNAMICS OF LIPOSOMES

To allow for an unambiquous interpretation of the data on the dissociation of cyt *c* from LUVs by these drugs, we first assessed the changes in pyrene fluorescence due to their binding to PPDPG containing liposomes in the absence of cyt *c*. For intermolecular excimer forming probes such as PPDPG changes

in I_e/I_m can be due to altered lateral diffusion, changes in the lateral distribution of the fluorescent probe, or both. In order to distinguish between these possibilities we measured the corresponding changes in fluorescence anisotropy (r) for the rod-like hydrophobic probe, DPH, incorporated into liposomes (Macdonald et al., 1988). In general, increase in r can be expected to mirror increase in acyl chain order of the membrane, which in turn attenuates lateral diffusion of lipids. The latter should be evident as decreased I_e/I_m. Accordingly, under conditions where both r and I_e/I_m increase the latter parameter is likely to reflect lateral enrichment of the pyrene-labeled lipid (Rytömaa & Kinnunen, 1996). These experiments were conducted both at X_{PG}=0.20 and 1.00 so as to further compare their effects on the A- and C-site lipid association of cyt *c*, respectively. To point out the possible coupling of these two parameters r **vs**

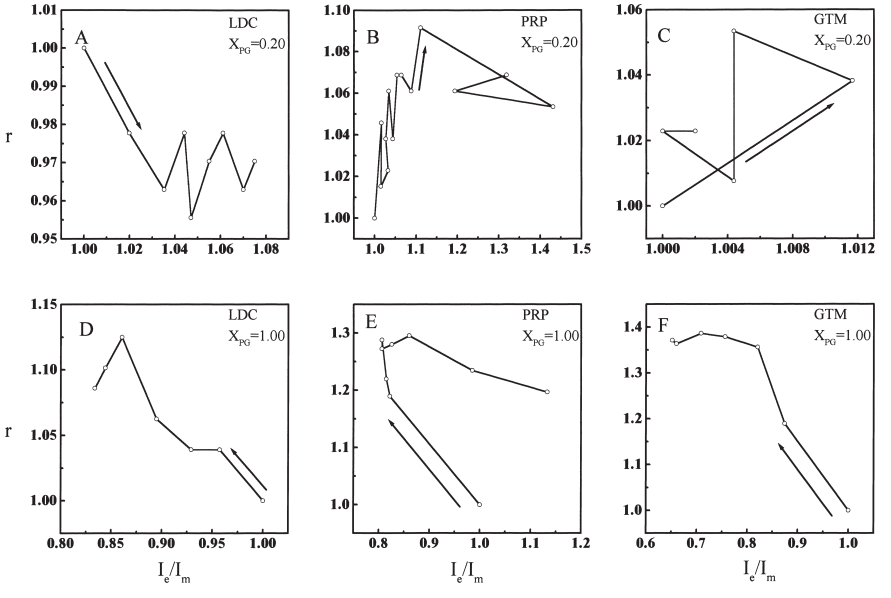

FIGURE 4. *The coupling of fluorescence anisotropy of DPH and excimer formation of PPDPG at X_{PG}=0.20 (upper row) and 1.00 (lower row) at various LDC, PRP, and GTM (from left to right) concentrations. The applied drug concentrations were those used in the measurements monitoring dissociation of cyt c from the membranes, and the directions of increasing drug concentrations are indicated by the arrows.*

I_e/I_m at varying drug concentrations is plotted in Fig. 4.

At $X_{PG} = 0.20$ increasing LDC concentration up to 10 mM progressively augments excimer formation, whereafter saturation is reached with an approx. 7 % increase in I_e/I_m (Fig. 4A). However, the opposite is true for r, thus indicating an increase in membrane free volume, and consequently the rate of lipid lateral diffusion. More pronounced effect on I_e/I_m, an increase by 42 %, was observed at 20 mM PRP (Fig. 4B). Further increase in [PRP] up to the highest concentration studied, 34 mM, enhanced I_e/I_m linearly (data not shown). Increase in I_e/I_m is paralleled by an increase in r, thus revealing PPDPG to become enriched into microdomains. Since GTM (up to 63 μM) has no effect on I_e/I_m, and also the changes in r are maximally ≈ 5 %, no correlation was observed in this case (Fig. 4C).

At $X_{PG} = 1.00$ the effects of these drugs were strikingly different. A decrease in I_e/I_m by approx. 15 % was observed for 15 mM LDC (Fig. 4D), whereas 20 % decrease was caused by 3 mM PRP (Fig. 4E). However, for PRP this decrease was followed by a subsequent linear increase, similarly to the effect of this drug at $X_{PG} = 0.20$. Interestingly, at $X_{PG} = 1.00$ also GTM decreased I_e/I_m by approx. 35 % (Fig 4F). The latter effect was evident already at 20 μM concentration of this drug. At $X_{PG} = 1.00$ and at low concentrations all three drugs increased fluorescence anisotropy of DPH. Accordingly, the attenuation of excimer formation is at least partly caused by diminished lateral diffusion caused by these drugs. Yet, at [LDC] $>$ 6 mM anisotropy decreases, thus indicating that the observed further decrease in I_e/I_m results from lateral enrichment of PPDPG. The same pattern was evident also for GTM which in concentrations exceeding 6 μM has no effect on r. At [PRP] $>$ 3 mM the increase in I_e/I_m is accompanied by decreased DPH anisotropy. However, this decrease in r is not as pronounced as the increase evident at lower PRP concentrations (i.e. $<$ 3 mM), thus indicating that the increase in I_e/I_m caused by this drug is only partly due to an augmented lateral diffusion of PPDPG.

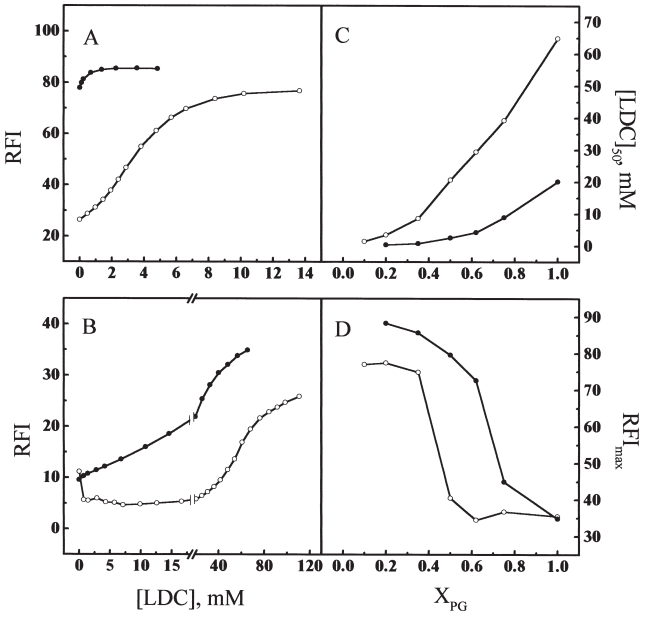

FIGURE 5. *Dissociation of cyt c from LUVs by lidocaine at X_{PG}= 0.20 (panel A) and 1.00 (panel B). Open and solid symbols indicate the absence and presence of 5 mM ATP, respectively. Panel C shows [LDC] producing 50 % recovery of RFI, and panel D maximal recovery of RFI by LDC as a function of X_{PG}.*

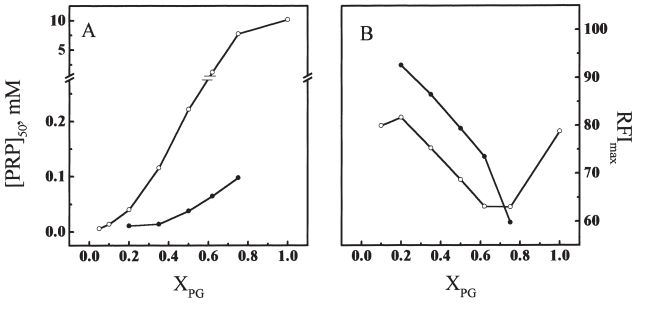

FIGURE 6. *The effect of lipid composition and ATP on the ability of propranolol to dissociate cyt c from liposomes are illustrated as $[PRP]_{50}$ and RFI_{max} in the absence (o) and presence (•) of 5 mM ATP at various X_{PG}.*

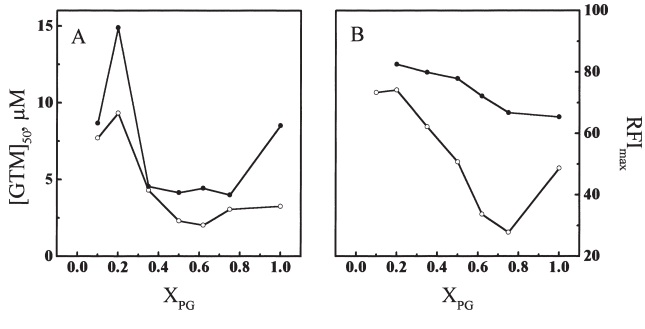

FIGURE 7. *The effect of lipid composition and ATP on the ability of gentamycin to dissociate cyt c from liposomes,expressed as $[GTM]_{50}$ and RFI_{max} in the absence (o) and presence (•) of 5 mM ATP at various X_{PG}.*

THE ABILITY OF LDC, PRP AND GTM TO DISSOCIATE CYT C FROM LIPOSOMES DEPENDS ON THE CONTENTS OF ACIDIC LIPID AND ATP

Electrostatic interactions are critically involved in the binding of cyt *c* to acidic phospholipids. Accordingly, it could be readily anticipated that similarly to the effect of sphingosine (Mustonen et al., 1993) also cationic, membrane partitioning drugs should interfere with the lipid binding of cyt *c* and eventually dissociate this protein from liposomes.

At $X_{PG} = 0.20$ LDC in a concentration of 8 mM reversed the A-site interaction of cyt *c* with acidic phospholipids, with maximally approx. 80 % recovery of the initial fluorescence intensity (Fig. 5A). At $X_{PG} = 1.00$ LDC concentrations up to 110 mM increased relative fluorescence intensity (RFI) from 8 to maximally 35 (Fig. 5B). Data from measurements similar to those illustrated in Fig. 5A and 5B, were subsequently collected so as to quantitate $[LDC]_{50}$ and RFI_{max} as a function of X_{PG}, i.e. drug concentrations required for half-maximal reversal of the quenching of pyrene fluorescence by cyt *c* (Fig. 5C) and the extent of maximal recovery of fluorescence (Fig. 5D), respectively. Upon increasing X_{PG} from 0.20 to 1.00 $[LDC]_{50}$ increases approx. 20-fold in the absence and 50-fold in the presence of 5 mM ATP. The ability of LDC to detach cyt *c* from membrane is strongly reduced when $X_{PG} \geq 0.50$, as shown by decreased values for RFI_{max}, thus indicating a change in the nature of either cyt *c*-phospholipid or LDC-phospholipid interaction or both at this liposome composition. This change is likely to arise from different lipid packing below and above this mole fraction of the acidic phospholipid. The potency of PRP to detach cyt *c* from vesicles exceeds that of LDC, especially at higher contents of PG. More than 75 % of the initial fluorescence is recovered by this drug at $X_{PG} \leq 0.50$ and at $X_{PG} = 1.00$ (Fig. 6B). However, the efficiency of this drug to detach cyt *c* is lower when $0.50 \leq X_{PG} \leq 0.75$. An apparently exponential dependency $[PRP]_{50}$ **vs** X_{PG} is evident both in the absence as well as in the presence of 5 mM ATP (Fig. 6A).

In order to compare the contributions of hydrophobic and electrostatic forces to the drug-membrane-interactions, experiments similar to those described above for LDC and PRP were subsequently carried out with GTM (Fig. 7).

The values for $[GTM]_{50}$ required for half-maximal reversal of quenching were significantly lower than those of LDC, 9.3 and 3.2 μM at $X_{PG} = 0.20$ and 1.00, respectively. However, the extent of the reversal was less complete, in particular at higher contents of acidic lipid, RFI_{max} varying between 25 and 75.

Because the effects of GTM deviated from those of the two amphiphilic drugs we also studied the binding of cyt *c* to liposomes subsequent to the prior additions of 60 μM and 6 μM GTM at $X_{PG} = 0.20$ and 1.00, respectively. Interestingly, under both conditions only about 25 % decrease in fluorescence intensity was observed upon increasing [cyt *c*] up to one μM. Accordingly, although GTM lacked effect on lipid dynamics at $X_{PG} = 0.20$ when investigated by I_e/I_m and DPH polarization, also under these conditions this drug must strongly bind to the liposome surface.

ATP augmented the detachment of cyt *c* by LDC and PRP, especially at PG contents with A-site binding contributing to the interaction, resulting in lower values of $[drug]_{50}$ and higher RFI_{max}. For GTM ATP increased RFI_{max} at all values of X_{PG}, but in contrast to what is observed for the two amphiphilic drugs ATP increased $[GTM]_{50}$.

However, at $X_{PG} = 1.00$ low concentrations of the studied drugs added subsequently to cyt *c* actually caused a further decrease in emission intensity indicating RET between pyrene and the heme of cyt *c* to become more efficient. In the presence of ATP this phenomenon was not observed.

THE EFFECTS OF CLZ, CPZ AND HPD ON THE PHASE TRANSITION OF LIPOSOMES

Binary phospholipid mixture of zwitterionic DPPC and acidic brainPS ($X_{PS} = 0.05$) was chosen as the target membrane for the neuroleptic drugs CLZ, CPZ, and HPD. In the endotherm for this content of brainPS a separate small peak/shoulder remains at T_m for neat DPPC vesicles (~ 41.3 °C) suggesting a presence of domains practically devoid of PS together with mixed domains containing both PC and PS (Fig. 8). This can be rationalized as follows. Electrostatic repulsion between the negatively charged PS headgroups resists the formation of domains enriched in this lipid, with even distribution representing

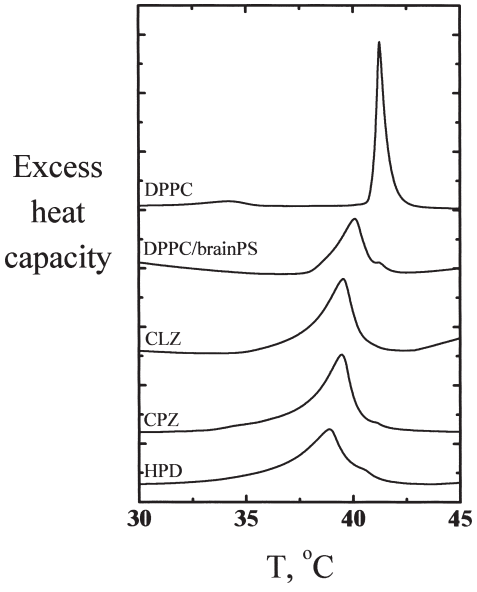

FIGURE 8. *The effects of brainPS and the neurolepts on the phase transition of DPPC. Endotherms of DPPC, DPPC/brainPS (95/5), and DPPC/brainPS/drug (90/5/5) MLVs. Scanning rate was 0.5 degrees/min, and the total lipid concentration was 0.4 mM.*

the free energy minimum. However, at $X_{PS} \geq 0.05$ this repulsion is partly overcome by an attractive potential, most likely due to hydrogen bonding between the PS headgroups (Boggs, 1987), similarly to that suggested for another acidic lipid, phosphatidylglycerol (II).

One of the major goals was to compare the atypical neuroleptic CLZ with two conventional neuroleptics CPZ and HPD in their interactions with phospholipid membranes. Importantly, grouping into atypical and conventional neuroleptics is based on their clinical response and receptor binding profile, not chemical structure. Both CPZ and HPD have been reported to partition into DPPC liposomes (Hanpft & Mohr, 1985; Sarmento et al., 1993). In order to investigate the contribution from electrostatic and hydrophobic interactions to the binding of CLZ to the bilayer, we first studied the effect of increasing contents of this drug on the thermal phase behaviour of DPPC liposomes. Increasing [CLZ] was found to decrease gradually the temperature as well as DH values for both pre- and main transitions revealing CLZ to bind to DPPC liposomes. As the latter is zwitterionic the interaction is likely to be driven by hydrophobicity.

The impact of the acidic PS to drug-membrane interactions as well as the differences in the effects of these drugs were explored in experiments utilizing

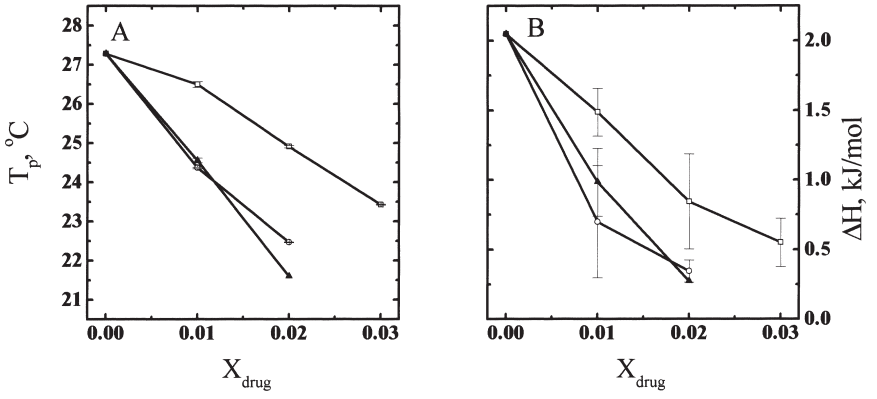

FIGURE 9. *Effects of increasing CLZ (❑), CPZ (❍) and HPD (▲) content on temperature (Panel A) and enthalpy change (Panel B) of the pretransition of DPPC/brainPS MLV's at* X_{PS} = *0.05.*

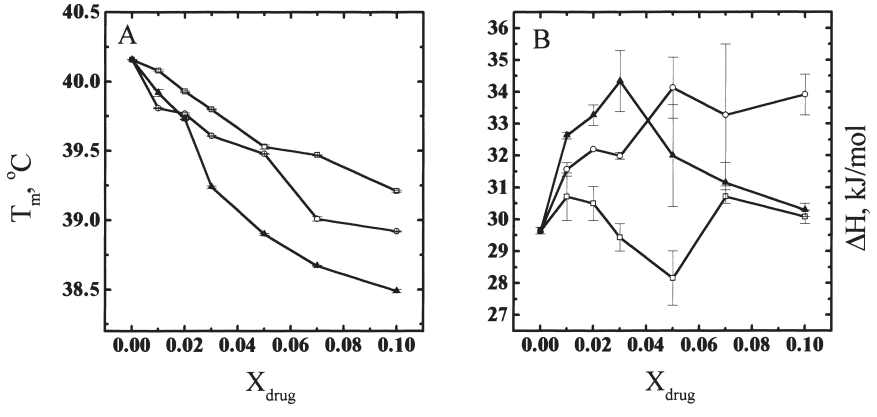

FIGURE 10. *Effects of increasing CLZ (❑), CPZ (❍) and HPD (▲) content on temperature (Panel A) and enthalpy change (Panel B) of the main transition of DPPC / brainPS MLV's at* X_{PS} = *0.05.*

DPPC/brainPS (95/5, mol/mol) MLVs. Interestingly, at X=0.05 CLZ abolished the peak at ~ 41.2 °C (Fig. 8), while in the presence of CPZ and HPD phase separation was observed up to the highest concentration (X_{drug} = 0.10) of the drug studied (data not shown). The latter could be related to charge neutralization of brainPS by the latter two compounds and enrichment of these drugs into the brainPS/DPPC domains. The different effect of CLZ suggests the hydrophobic interactions of this compound to be more important for membrane

association than for CPZ and HPD. Moreover, our data indicate CLZ to partition into the thermally more stable DPPC enriched domains.

All three drugs decreased gradually both T_p and ΔH of pretransition, the effects being least for CLZ (Fig. 9). At $X_{CLZ} > 0.03$, and at X_{CPZ} and $X_{HPD} > 0.02$ pre-transition was not observed. With increasing drug concentrations values for T_m shift gradually towards lower temperatures (Fig. 10). Enthalpy of the main transition is increased up to $X_{drug} = 0.02$ for all three drugs, this enhancement being largest (approx. 16 %) for HPD. At higher X_{drug} the effects become dissimilar, as follows. For CLZ, the enthalpy remains approx. equal to that measured in the absence of the drug, the only exception being $X_{CLZ} = 0.05$, i.e. when the acidic phospholipid:CLZ stoichiometry is 1:1, where ΔH has a minimum of 26.5 kJ/mol (Fig. 10B). At $X_{CLZ} = 0.05$, there is also a change in the dependence of T_m on X_{drug} (Fig. 10A). For CPZ, further increase in X_{CPZ} has only a minor effect on variation in ΔH, with maximal increase of ~4.4 kJ/mol (~ 15 %). For HPD, at $X > 0.02$ ΔH decreases almost linearly reaching a minimum of 30.3 kJ/mol at $X_{HPD} = 0.10$.

CLZ, CPZ AND HPD BIND, PENETRATE AND CHANGE THE DOMAIN MORPHOLOGY OF LIPID MONOLAYERS

COMPRESSION ISOTHERMS

Phospholipid monolayers provide a highly informative approach for studying drug-lipid interactions as the lipid composition has no effect on the surface curvature and the lateral packing can be precisely controlled (Brockman, 1999). Compression isotherms revealed all three drugs to increase the area/acyl chain for DPPC/brainPS monolayers (Fig. 11). At the surface pressure range for the liquid-condensed region (for a recent review, see Kaganer et al., 1999) an approx. constant increase in surface area is caused by the drugs, in keeping with their tight and pressure dependent association to the lipid films (Fig. 11 inset). At 20 mN/m and in the absence of the drugs this value was 22.3 $Å^2$, and was increased up to 25.3, 24.1, and 23.4 $Å^2$ by CLZ, CPZ, and HPD, respectively. Due to the high affinity for PS of these drugs we may assume their partitioning into the subphase to be negligible and calculate the mean molecular areas

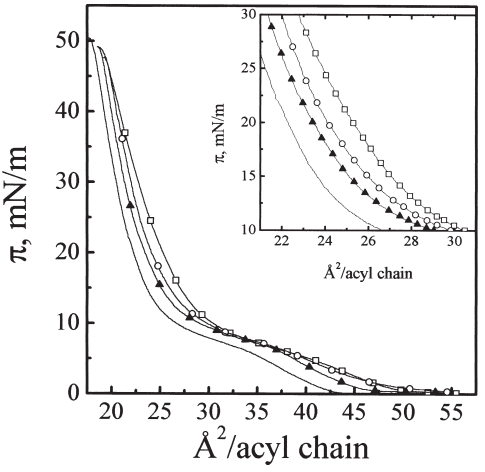

FIGURE 11. *Compression isotherms of DPPC/brainPS/drug monolayers at X_{PS} = X_{drug} = 0.05 for CLZ (❑), CPZ (○), and HPD (▲). The line without symbols corresponds to the DPPC/brainPS (0.95/0.05, mol/mol) monolayer. The inset shows an enlargement of the surface pressure range in liquid expanded (LE) - liquid condensed (LC) coexistence region. Monolayers were compressed continuously at a rate of one Å²/acyl chain/min.*

to be 114, 68, and 42 Å² for CLZ, CPZ, and HPD, respectively. Interestingly, in the liquid expanded-liquid condensed coexistence region (at p ~ 8-9 mN/m) the isotherms for the drug containing monolayers were almost superimposable and the mean molecular areas were approximately 137 Å² for CLZ and CPZ, and 114 Å² for HPD.

BINDING OF THE NEUROLEPTICS TO MONOLAYERS

All the three neuroleptics were found to increase the surface pressure ($\Delta\pi$) of both neutral eggPC and acidic brainPS monolayers residing at air-buffer interface after injecting of the drug into the subphase (Fig. 12). The initial surface pressure (π_0) was varied in the range of approx. 12-35 mN/m. Most efficient penetration into eggPC monolayers was evident for CLZ which under the conditions used induced $\Delta\pi$ of about 3 mN/m at π_0= 10-25 mN/m, whereas the other two drugs interacted more weakly, with $\Delta\pi$ being 1-2 mN/m. Penetration of the drugs into eggPC decreased with increasing π due to augmented lipid lateral packing. Critical surface pressures abolishing the penetration of CLZ, CPZ, and HPD were ~40, 32, and 25 mN/m, revealing the atypical neuroleptic CLZ to be the most membrane active of these compounds in neat PC monolayers. These results support the conclusion drawn from DSC and from compression isotherms that for clozapine hydrophobic interactions are more im-

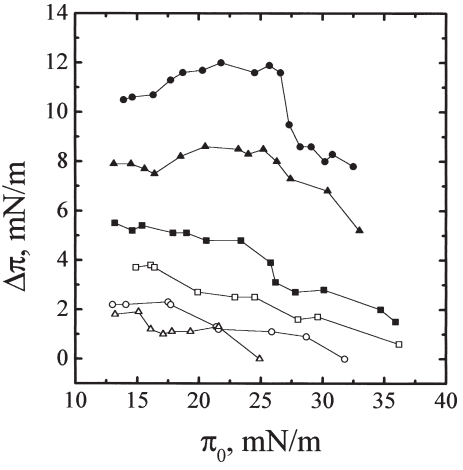

FIGURE 12. *Penetration of neurolepts into monolayers as indicated by increase in the surface pressure (Dp) of brainPS (solid symbols) and eggPC (open symbols) monolayers at different initial surface pressures (p_0) upon injection of final concentration of 1.3 μM CLZ (■,❑), CPZ (●, ❍), or HPD (▲, Δ) into the subphase.*

portant for its membrane association than for chlorpromazine and haloperidol. The effects of the drugs on brainPS monolayers were more pronounced than for eggPC. At π_0= 12-25 mN/m, $\Delta\pi$ for brainPS monolayer was approx. 5, 11, and 8 mN/m by CLZ, CPZ, and HPD, respectively. At π_0 ~ 26 mN an abrupt decrease in $\Delta\pi$ was observed. This could reflect augmented protonation of the PS headgroups at higher packing densities with subsequent hydrogen bonding resulting in enhanced PS-PS interactions (Boggs, 1987), impeding the penetration of the drugs driven by electrostatic attraction.

FLUORESCENCE MICROSCOPY

Fluorescence microscopy images of DPPC/brainPS/NBD-PC (93/5/2, mol/mol) monolayer are characterized by the presence of domains at surface pressure values above 12 mN/m (Fig. 13), revealing the two phase region of the monolayer, as suggested also by the discontinuity observed in the compression isotherms (Fig. 8). The differential 'staining' of the coexisting gel state and fluid domains is due to the efficient partitioning of the fluorescent probe NBD-PC into the fluid (i.e. liquid expanded) domains (Weis & McConnell, 1985). In the absence of the drugs, the domains are relatively large with a distinct roundish shape. The size of the domains is affected by all three drugs and the domain morphologies become more complex in the presence of CPZ and HPD.

Instead, only slight changes in domain morphology are caused by CLZ, while the average size of gel state domains decreases. Both of these effects increase the length of the boundary between gel and fluid domains, indicating that the drugs stabilize the boundary and thus enhance phase separation. This also suggests preferential partitioning of these drugs into the domain boundaries.

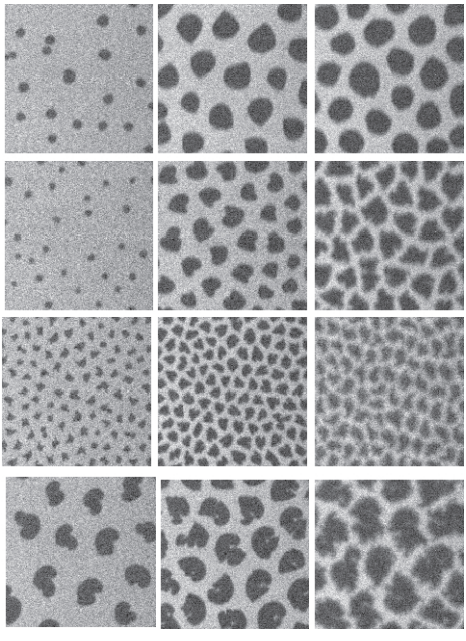

FIGURE 13. *The effects of the neurolepts on the domain morphology. Fluorescence microscope images of DPPC/brainPS/NBD-PC/drug (88:5:2:5) monolayers at 15, 20, and 25 mN/m (from left to right) after 10 min stabilization in the absence of drugs (uppermost panels) and in the presence of CLZ, CPZ, and HPD (from top to bottom).*

DISCUSSION

One of the re-occuring concepts in the present thesis is lateral organization of lipid membranes; how it can be triggered and controlled, and how does it effect the functions of the membrane. The focus of the first contribution of the thesis is the organization of lipid bilayer in the course of the main phase transition, where lateral heterogeneity in the membrane is induced by changing the ambient temperature (I). The accompanying studies indicate that composition and lateral organization of lipid bilayer controls the rates for attachment and detachment and the mode of interaction between vesicles and proteins cyt *c* and H1 (II). Furhermore, it is shown that widely used pharmacological agents (LDC, PRP, GTM) have high affinity to phospholipid membranes, and subsequently are able to trigger the changes in the lateral organization required for detachment of cyt *c* (III). In (IV) the effects of three neuroleptic drugs (CLZ, CPZ, HPD) on lateral heterogeneity are compared by DSC and monolayer techniques, and visualized by fluorescence microscopy.

Fluorescence spectrocopy is a widely used method in the field, and can provide insight both into the structure and dynamics of membranes. However, there are several issues to be considered when interpretating fluorescence data, as follows. (i) Fluorescence lifetime of the utilized probe sets the time window for the processes that can be monitored. Considering the present study, the lifetime of pyrene is relatively long, of the order of 10^{-8} sec, and during this time the excited probes can diffuse laterally within the membrane and possibly form excimers or undergo RET. Thus, this probe can provide information on the membrane dynamics on a long time scale. On the other hand, fluorescence lifetime of DPH is one order of magnitude shorter, and consequently this probe would provide 'snapshots' of the physical state of the membrane and acyl chain conformational dynamics. (ii) The vertical location of probe in the membrane affects its microenvironment, and consequently also its photophysics. In brief, the polarity is higher in the vicinity of the headgroup region

of the bilayer. On the other hand, if the probe is covalently linked to the hydrocarbon chain of a lipid molecule, its motional freedom is higher the deeper in the hydrophobic core the linkage is. (iii) In many cases the lateral distribution of the probe is not homogeneous in the membrane, but instead it favors either fluid or gel domains or their interfacial boundary. Consequently, in the phase co-existence region the fluorescence data provides information mainly on the properties of the favored phase.

MAIN PHASE TRANSITION

In the transition region the coexisting phases undergo intense fluctuations, as recently shown by atomic force microscopy of phospholipid monolayers deposited on mica sheets (Nielsen et al., 2000). The present results provide evidence for lateral heterogeneity in DMPC LUVs below T_m, as a transient increase in excimer formation is observed for the pyrene labeled lipid PPDPC (Fig. 2). The resonance energy transfer data support the view that the transient peak in I_e/I_m at T* originates from a fraction of PPDPC in the membrane partitioning into the interfacial boundary separating "fluid" domains from the gel bulk. The driving forces for the enrichment of PPDPC into the interface below T_m can be rationalized as follows. As a substitutional impurity PPDPC perturbs the gel state lattice and it can be anticipated to be repelled, although weakly, from this matrix, similarly to the exclusion of this probe from the gel state of DPPC (Somerharju et al., 1985). The boundaries between the different phases are not exact, sharp lines, but gradients in which the rate and the extent of *trans* → *gauche* isomerization change (Hwang et al., 1995). In accordance, phase boundaries are "soft" and easily accommodate impurities (Mouritsen et al., 1995). On the other hand, upon main transition, there is a ~20 % reduction in bilayer thickness and ~20 % lateral area expansion (Wilkinson & Nagle, 1981). As the effective length of PPDPC exceeds the thickness of fluid DMPC (Lehtonen et al., 1996), hydrophobic mismatch opposes the partitioning of the probe also into the fluid phase. While increase in free energy due to hydrophobic mismatch between PPDPC and gel state DMPC should be less than that between PPDPC and fluid phase DMPC, the perturbation by PPDPC of

the packing of DMPC in the gel state is more severe than that imposed by the probe on the packing of fluid phase DMPC. A free energy minimum appears to be achieved when a fraction of PPDPC is localized into the boundary, thus resulting in a local enrichment of the probe and augmented excimer formation.

The formation of fluid domains and the interface starts already at T_0 (≈20 °C), well below T_m (Fig. 2), as evidenced by steeper increase in I_e/I_m due to the enrichment of PPDPC into the boundary. Upon further heating in the interval $T_0 < T < T^*$, more domains appear increasing the total length of the boundary and I_e/I_m due to a larger number of PPDPC becoming accommodated into the interface. Importantly, the temperature for the I_e/I_m maximum was not shifted when X_{PPDPC} was increased from 0.01 to 0.03. This observation contradicts the view that the decrease in I_e/I_m above T* would be due to an increase in the length of the boundary and local dilution of the probe. Instead, these data supports the interpretation of the boundary length having a maximum at T*, whereafter the decrease in excimer formation would report shortening of the boundary.

Hresko et al. (1986) have reported PPDPC to partition equally into fluid and gel domains in DMPC vesicles, but favor fluid domains in a DPPC matrix. This result is reasonable if we consider the thickness of the membrane (DPPC>DMPC) and hydrophobic matching of the probe into the fluid domains. However, that study did not consider the presence of boundaries at all, and utilized small sonicated vesicles. Importantly, it points out that the same probe can exhibit different lateral distribution in different matrices. Thus, this is not necessarily in conflict with the present study indicating PPDPC to be weakly enriched into domain boundary in DMPC matrix.

Above T_m the minor deviation from the baseline (Fig. 2B) suggests that PPDPC is weakly enriched into the boundary also in this temperature range. Importantly, the physical properties of fluid domains in the dominantly gel state bilayer below T_m are not identical to those of the fluid areas above T_m. This is understandable as upon increase in T, the extent and rate of *trans* → *gauche* isomerization of the acyl chains in fluid domains further increases, in other words fluid domains become "more fluid". Simultaneously, also the properties of the remaining gel phase change in an analogous manner, and the phys-

ical properties of the gel domains in the fluid bilayer above T_m are not representative of the gel state below T_m. Accordingly, the properties of the boundaries change in the course of the main phase transition.

The microdomain formation is evident below and above the thermal transition, but at T_m the boundary appears to vanish completely on the time scale of the pyrene excimer lifetime, as indicated by (i) I_e/I_m and by (ii) a minimum in C at T_m for all three quenchers. The latter data reveal that there is a maximum in the average distances between PPDPC excimers and the different acceptors at T_m. The absence of domains and their boundaries necessitates the nature of the fluctuating entities underlying the maximum in heat capacity at T_m (Doniach, 1978; Freire & Biltonen, 1978; Mouritsen et al., 1995) to be reconsidered.

Interpretation of the present results requires a mechanism for the main transition involving two subsequent steps in the vicinity of T_m: gel $\leftrightarrow$ intermediate and intermediate $\leftrightarrow$ fluid, as follows. With increasing temperature of a gel state lipid lattice well below T_m the number of separate lipids with acyl chains in gauche conformation first increases (Kosterlitz & Thouless, 1973). Nucleation of fluid domains by these thermally excited lipids commences at T_0 and results in the augmented I_e/I_m due to the weak enrichment of PPDPC into the domain boundaries. The fluid domains subsequently increase in their size and number, and the length of the boundary is maximal at T*. Close to T* the fluid domains coalesce to form a continuous phase, and subsequently the total boundary length begins to decrease. Upon further increase in temperature the transition is not directly from the gel into the fluid phase, however, and these phases are separated by an intermediate phase existing at temperatures close to T_m. Accordingly, at proper thermal excitation ($T^* < T < T_m$) the domains merge into a highly cooperative lattice. To some extent this represents a situation where the entire bilayer has the properties of a fluctuating gel/fluid interface. Within a narrow temperature interval centered at T_m the bilayer would thus consist of a fluctuating, extremely cooperative superlattice (Kinnunen, 1991; Somerharju et al., 1985; Kinnunen et al., 1987; Tang & Chong, 1992; Sugar et al., 1994; Chong et al., 1994) of regularly distributed fluid state phospholipids, ideally forming a lattice of 1:1 stoichiometry with gel state phospholipids. Above T_m transition from the intermediate phase to gel domains in

fluid bulk takes place. Upon further increase in T, the size and number of the gel domains decrease, the entire membrane becoming fluid.

A number of alternative models explaining the decline of excimer formation in below T_m should also be considered, as follows. Excimer formation and resonance energy transfer are sensitive to the mutual orientation of the two probes, and non-parallel orientation, due to e.g. steric or hydrophobic hindrances creating a high energy barrier, would result in a drop in I_e/I_m and C values. In the transition process the membrane thickness decreases, that is the fluid domains are thinner than the gel state region. In the early stage of the transition the fluid domains are small, and do not necessarily induce a fluid domain also in the opposing leaflet in the same lateral location, i.e. the domain formation is asymmetrical. For PPDPC in this asymmetric fluid domain hydrophobic mismatch has to be considered, especially in DMPC matrix, as it cannot penetrate into the opposing gel state leaflet or extend out of the membrane into the water phase. These hydrophobic and steric hindrances could force the long probe molecule to tilt into a conformation unfavorable for excimer formation. At higher temperatures the fluid domains grow in size, and consequently lateral heterogeneity becomes symmetrical. This enables PPDPC to interdigit into the opposing now fluid leaflet and avoid tilting. In this model, further increase in I_e/I_m could follow also from possible interlayer excimerization between pyrene-labeled lipids residing in the opposing leaflets of the bilayer. Another alternative might be the existence of several pyrene sub-populations, some of them in an microenvironment not favorable for excimer formation (Sugar et al., 1991). Importantly, these explanations for the decline of I_e/I_m are not exclusive, but more than one of these phenomena may contribute to the observed effect.

KINETICS OF THE LIPID-PROTEIN ASSOCIATION

In liposomes the degree of protonation of the acidic phospholipid increases with its mole fraction, i.e. with increasing electrical potential (Träuble, 1976). Due to coulombic repulsion the distances separating deprotonated PGs bearing negative charge should be maximal, and clusters of deprotonated acidic

phospholipids should not be present in the bilayer. Upon increasing X_{PG} from 0.20 to 0.40 the surface net negative charge and the number of binding sites for cationic proteins in the membrane surface increases (Rytömaa & Kinnunen, 1994). However, the present data reveal that upon increasing X_{PG} the binding of cyt *c* and H1 becomes slower, i.e. the affinity of the vesicle surface for these proteins is diminished. This is unexpected as increasing negative surface charge density would be anticipated to enhance coulombic attraction and thus to accelerate the membrane association. As the converse is true it follows that an energy barrier dependent on X_{PG} must be involved which retards the binding for both proteins. Such barrier could be provided by highly cooperative hydrogen bonded networks formed by deprotonated and protonated PG (Watts et al., 1978; Eibl & Blume, 1979; Boggs, 1987; Haines, 1983; Garidel et al., 1997) which would further stabilize the lateral distribution of deprotonated PG molecules in the membrane. The distribution of charges appears to be critically dependent on X_{PG} and different types of lipid headgroup arrays appear to be formed below and above $X_{PG} = 0.50$. The lipid domains providing the binding sites for cyt *c* are stabilized by increasing content of the acidic phospholipid, as is clearly evident from the decreasing rates upon increasing X_{PG}. Additional stabilization could be due to the proposed extended lipid anchorage of cyt *c* to lipid vesicle surface (Kinnunen et al., 1994; Rytömaa & Kinnunen, 1995; Kinnunen, 1991), as described in the Review of the literature. In most conditions the decay of fluorescence resulting from the membrane binding of the proteins was two-exponential. The most likely interpretation is that the initial fast binding process is followed by slow alterations in the membrane lateral order in a manner causing more of the pyrene-labeled probe to diffuse within the quenching radii of the proteins. Similar reasoning would also explain the slow changes in fluorescence following the initial rapid release of cyt *c* by NaCl, H1 and the other cationic ligands. Halftimes for the membrane association of FITC-H1 vary from 7.9 to 52 msec, at $X_{PG} = 0.20$ and 1.00, respectively. Accordingly, attachment of H1 to LUVs in the presence of cyt *c*, as indicated by the dissociation of the latter, are significantly retarded, with the corresponding halftimes of 17 and 204 msec. This is readily comprehensible as charges in the membrane surface should be neutralized by the bound cyt *c*, thus reducing the electrostatic attraction of H1 to the vesicle surface.

Notably, at $X_{PG} \geq 0.50$ also the mode of lipid-cyt *c* interaction becomes different as demonstrated by the loss of the ability of ATP to detach the protein. The C-site mediated binding of cyt *c* measured in the presence of ATP and varying X_{PG} between 0.50 and 1.00 is single-exponential, the fast component being absent. Interestingly, the rate of this process decreases upon increasing $X_{PG.}$ Analogously to the discussion above the reason for this could be intermolecular hydrogen bonding between PG headgroups competing for the interaction with cyt *c*. ATP appears to induce a change in the conformation of cyt *c* bound to membrane via its C-site (Rytömaa et al., 1992; Tuominen et al., 2001). Accordingly, the difference in the half-times measured with X_{PG} in the range of 0.50 to 1.00 and in the absence and presence of ATP is not unexpected. Conversely, these data strongly support the concept of the fast process measured in the absence of ATP to represent electrostatically driven binding of cyt *c* to liposomes.

Similarly to ATP, NaCl should release only the A-site bound cyt *c*. However, dissociation of the protein from the vesicles by NaCl was evident even at $X_{PG} = 1.00$. This indicates that the addition of NaCl reduces the protonation of PG, alters lateral lipid distribution in the bilayer (Rytömaa et al., 1992; Rytömaa & Kinnunen, 1994; 1995; Träuble, 1976), and eventually the binding mode of cyt *c* changes from C-site to A-site association.

The detachment of cyt *c* from LUVs by H1 and the cationic membrane-binding peptides myr-KRTLR, ACTH 1-24, and K_{19} are similar in that for all of them the increase in fluorescence due to the release of cyt *c* from liposomes was two-exponential. Likewise, the fast and the slow process were both attenuated upon increasing X_{PG}. Although the concentrations of the ligands were such that they all produced saturating responses in steady-state measurements there were also marked differences in the dissociation of cyt *c* by them. It seems likely that similarly to the binding of cyt *c* to membranes containing acidic phospholipids also for H1 the association of H1 to membranes involves hydrogen bonding between protonated PG and H1. The differences in the release of cyt *c* by these ligands are likely to reflect differences in the relative contributions of hydrophobicity, coulombic attraction, and hydrogen bonding in causing their attachment to the vesicles.

EFFECTS OF THE DRUGS ON MEMBRANES

Changes in the membrane lateral heterogeneity are caused by all six drugs studied in this work. Importantly, the effects of these drugs are not identical, indicating that the observed changes are not caused by non-specific interactions. It should be emphasized that drug induced changes in lipid membrane lateral heterogeneity are dependent on membrane lipid composition, the compound itself, and factors such as temperature, pH, osmolarity, and ionic strength (Kinnunen, 1991), as recently demonstrated for cyclosporin A (Söderlund et al., 1999a).

LIDOCAINE, PROPRANOLOL, AND GENTAMYCIN

Binding of LDC, PRP, and GTM to liposomes influenced lipid dynamics as judged by changes in the efficiency of excimer formation of PPDPG as well as in fluorescence anisotropy of DPH (Fig. 4). In brief, at X_{PG}= 0.20 LDC enhanced lipid lateral diffusion while GTM had no effect. On the other hand, PRP rigidified the membrane and caused lateral enrichment of PPDPG. At X_{PG}= 1.00 low concentrations of all the three drugs decreased lipid lateral diffusion, while at higher [drug] lateral enrichment of PPDPG was evident. For polycationic GTM aggregation of acidic phospholipids on the surface of liposomes is likely to be involved. This would cause formation of PG enriched domains, rigidification of bilayers, and decrease in lipid lateral diffusion, as indicated by the observed decrease in I_e/I_m. This effect weakens drastically upon decreasing X_{PG}, thus revealing the affinity of GTM to be strongly dependent on the content of the acidic phospholipid.

The present data suggests that the dissociation of cyt c from the membranes by LDC and PRP results mainly from charge neutralization of the acidic phospholipid. Consequently, the mechanism of the detachment varies with X_{PG}, and lateral organization of the lipids. At low X_{PG} the number of charges due to deprotonated PG in the bilayer is reduced by these drugs and, accordingly, the A-site bound cyt *c* is released from the surface. The electric potential of the membrane increases with X_{PG}, resulting in enhanced protonation of the acidic lipid (Träuble, 1976). In order to detach cyt *c* bound to the protonated PG via

the C-site of this protein, the cationic drugs must first bind to liposomes so as to decrease negative surface charge density and thus deprotonate PG. In the presence of the amphiphilic cationic drugs the mode of interaction between cyt *c* and acidic phospholipids is altered from C-site to A-site binding, even at X_{PG}= 1.00. Simultaneously, the liposome-associated drug competes with cyt *c* for binding the anionic lipid, thus releasing the protein from the bilayer. Also at X_{PG}= 1.00 the A-site binding is inhibited by ATP, and accordingly, in the presence of ATP all cyt *c* associated with the bilayer via the A-site is released from the membranes. In the light of the above, it seems feasible that the differences in the drug concentrations required for half-maximal reversal of the quenching $[drug]_{50}$ observed for the three compounds in the presence and in the absence of ATP mirror the different efficiencies of these drug to deprotonate the acidic phospholipid.

The hydrophobicity of GTM is significantly lower than those of LDC and PRP, and thus electrostatic attraction provides the main driving force for its membrane association (Brasseur et al., 1984). Compared to LDC and PRP remarkably lower concentrations of GTM suffice in releasing cyt *c* from liposomes. For instance, at X_{PG}= 0.20 the value for $[GTM]_{50}$ is approx. 20 % of $[PRP]_{50}$. The affinity of GTM to liposomes appears to increase with contents of acidic lipid, as indicated by the tendency of $[GTM]_{50}$ to decrease with inceasing X_{PG}. A plausible mechanism would be that the positively charged GTM readily ligands to deprotonated PG's effectively neutralizing the acidic phospholipids and thus blocks collisions of cyt *c* with the liposome surface.

As shown above binding of the cationic drugs induces deprotonation of PG and a shift in the binding mode of cyt *c* from C-site to A-site interaction at X_{PG} = 1.00. This was evident also as a further decrease in pyrene emission intensity (Fig. 5B), as energy transfer between PPDPG and cyt *c* is more efficient when the latter is bound via its A-site. In the presence of ATP these drugs did not decrease RFI, as the nucleotide inhibits the A-site binding of cyt *c*.

To conclude, the efficiency of these drugs to detach cyt c from the membranes is coupled to their potency to promote the deprotonation of the acidic phospholipids.

Yet, the charge of the deprotonated lipid is not neutralized by the drug, perhaps due to a higher affinity of the deprotonated PG for cyt *c* than the drug,

thus keeping the protein attached to membrane surface via the A-site of cyt *c*. In the presence of ATP, however, this interaction is blocked due to the binding of the nucleotide to the A-site. At X_{PG}= 1.00 LDC is not capable of promoting the deprotonation of PG and ATP has no effect on the membrane association of cyt *c*, the latter remaining bound to protonated PG via the C-site. GTM, instead, appears to effectively deprotonate PG also at X_{PG}= 1.00, thus enabling ATP to displace cyt *c* rather efficiently from liposomes.

CLOZAPINE, CHLORPROMAZINE, AND HALOPERIDOL

As shown in the fluorescence microscopy images (Fig. 13) all three neuroleptics increased the gel-fluid domain boundary length DPPC/brainPS monolayers, indicating decrease in line tension and/or increase in dipole repulsion (McConnell, 1991; Brockman, 1994; Perkovic & McConnell, 1997; Kaganer et al., 1999). These effects were stronger for the conventional neuroleptics, CPZ and HPD, than for the atypical neuroleptic, CLZ. Similar difference was also observed in DSC measurements, where CLZ abolished the phase separation in DPPC/brainPS MLVs, while CPZ or HPD did not. These findings indicate a striking difference in the effects of CLZ and the conventional neuroleptics on the heterogeneity of binary lipid mixture. Membrane permeability has been shown to correlate with domain boundary length (for a review see Mouritsen & Kinnunen, 1996), which could provide a mechanistic explanation for the observed increase in liposome permeability by CPZ (Maoi et al., 1979).

In keeping with DSC studies using DPPC MLVs, CLZ penetrated into egg-PC monolayers at significantly higher values of π_0 than CPZ or HPD and produced the largest $\Delta\pi$. Likewise, the lateral expansion of DPPC/brainPS (95/5) monolayers was largest for CLZ. The abrupt decrease in $\Delta\pi$ in the penetration of all three drugs into brainPS monolayers at ~25-26 mN/m could represent an attenuated affinity of the drugs towards the lipid monolayer as a consequence of diminished electrostatic attraction due to enhanced protonation of PS headgroups at higher surface charge densities. To conclude, electrostatic attraction seems to be important for the lipid binding of all three drugs, and additionally, for CLZ the contribution of hydrophobic forces is more stronger than for the conventional neuroleptics. The differences in the contribution of hydro-

phobic and electrostatic interactions on the membrane association of CLZ, CPZ, and HPD are likely to be contributing also to the observed changes in membrane heterogeneity caused by these compounds.

Mean molecular areas for these drugs calculated from the compression isotherms are in the same range (~114-137 Å^2/molecule) at low surface pressure (~9 mN/m) while at higher packing pressures (15-35 mN/m) the mean molecular area occupied by CLZ remains large (114 Å^2) whereas for CPZ and HPD a decrease to 68 Å^2 and 42 Å^2, respectively, is evident. This indicates the orientation of CLZ to be independent of the lateral pressure, while CPZ and HPD may reorientate and/or become excluded from the membrane interior at higher pressures. The latter possibility is indicated by the monolayer penetration measurements. Yet, the derived mean molecular areas as well as the compression isotherms suggest that in the surface pressure ranges generally considered to be relevant for biomembranes (approx. 30-35 mN/m) the degree of dissociation of the drugs from the monolayer should be neglible. The similar mean molecular area of ~114-137 Å^2 for the three compounds in the transition region is interesting. Notably, as the compressibility of the film has a maximum in the two phase region, this suggests that under these conditions the orientation of the compounds is solely controlled by the lateral pressure.

The current results on the interactions between neuroleptic drugs and phospholipid model membranes composed of binary lipid mixtures reveal qualitative differences between the two conventional neuroleptics, (CPZ and HPD) and atypical neuroleptic, CLZ. Accordingly, the two former compounds have a significant impact on membrane lateral heterogeneity, causing more pronounced rearreangements of the membrane constituents than the atypical neuroleptic CLZ. In the light of the above, it is tempting to suggest that the observed effects of CLZ, CPZ, and HPD on membrane lateral heterogeneity and PS-drug interactions could represent additional mechanisms by which these compounds may exert (part of) their pharmacological activities. Yet, more extensive comparison between atypical and conventional neuroleptics is required. Our findings also suggest possible use of membrane lateral heterogeneity for screening purposes in drug discovery.

CONCLUSIONS

Lateral heterogeneity is evident in the course of the main phase transition of DMPC lipid bilayer. In other words, at specific temperature ranges in the vicinity of the transition there exists gel and fluid domains and also probably superlattice structures. Furthermore, in the coexistence region pyrene-labeled lipid PPDPC was found to enrich into the interfacial boundary between gel and fluid domains.

Kinetics of binding and dissociation between cyt *c* and membrane is governed by the lipid composition of the latter. This is in accordance with cyt *c* having multiple lipid binding sites and the binding can be electrostatic or via hydrogen bonding depending on the content of acidic phospholipid in the membrane. The domains appear to be stabilized by formation of hydrogen bonded networks of protonated and deprotonated phosphoglycerols as the binding and dissociation of the proteins to liposomes becomes slower with increasing contents of acidic phospholipid. These data suggest that several functions of cell may be controlled by local structures in the lipid bilayer i.e. domains.

Dramatic changes in the structural dynamics of lipid bilayers were observed upon association with drugs lidocaine, propranolol and gentamycin. Furthermore, all the three drugs were able to dissociate peripheral protein cyt *c* from membrane. These results indicate an alternative way of pharmacological action for these drugs.

The three studied neuroleptic drugs clozapine, chlorpromazine and haloperidol associate with lipid membranes changing the thermal behaviour of vesicles and domain morphology of monolayers. In the membrane association the contribution of hydrophobic (vs. electrostatic) forces is more important for the atypical clozapine than for the conventional neuroleptics chlorpromazine and haloperidol.

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