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Lateral domain heterogeneity in cholesterol/phosphatidylcholine monolayers as a function of cholesterol concentration and phosphatidylcholine acyl chain length

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Abstract

Mixed monolayers of cholesterol and phosphatidylcholines having symmetric, different length acyl chains (10 to 16 carbons each) were prepared at the air/water interface. The partitioning of a fluorescent probe, NBD-cholesterol at 0.5 mol%, among lateral domains was determined by epifluorescence microscopy. The mixed monolayers had cholesterol concentrations of 20, 25, or 33 mol%, and in all these monolayers, lateral domain heterogeneity was observed within a defined surface pressure interval. This surface pressure interval was highly influenced by the phosphatidylcholine acyl chain length, but not by the cholesterol content of the mixed monolayer. The characteristic surface pressure, at which the line boundary between expanded and condensed phases dissolved (phase transformation pressure), and the monolayer entered an apparent phase-miscible state, was about 20 mN/m for di10PC and decreased as a linear function of the phosphatidylcholine acyl chain length to be about 2.5 mN/m for di16PC. During initial compression of the monolayers, the sizes of the condensed phases were generally larger, and to some extent heterogeneous with respect to the size distribution, as compared to the situation in monolayers which had experienced a compression/expansion cycle, which took them above the phase transformation pressure. This suggest that the domains observed during initial compression were not equilibrium structures. This study has demonstrated that both the cholesterol content and the phosphatidylcholine acyl chain length markedly influenced the properties of laterally condensed domains in these mixed monolayers. Since the possibility for the formation of attractive van der Waals forces between cholesterol and acyl chains increase with increasing acyl chain length, and since the phosphocholine head group is similar in all systems examined, the observed differences in domain shapes, properties, and stability most likely resulted from differences in van der Waals forces.

Keywords: Cholesterol; Phosphatidylcholine; Lateral domain heterogeneity; Monolayer; Epifluorescence microscopy; Lipid interaction

1. Introduction

Cholesterol and phosphatidylcholines constitute the major lipid classes of cellular plasma membranes. Their mutual interaction is of utmost importance for many essential membrane-associated functions, and their membrane behavior is governed by hydrophobic effects, van der Waals forces, and hydrogen bonding (at least to water molecules) [1–3]. The association of cholesterol with phosphatidylcholines is to a large extent determined by the acyl chain composition of the phospholipid, so that cholesterol form more densely packed membranes with saturated long-chain derivatives of phosphatidylcholines as compared with for instance shorter-chain phosphatidylcholines [4,5], or with phosphatidylcholines having a mono-unsaturated acyl chain at the *sn*-2 position [4]. This interaction between cholesterol and a phosphatidylcholine molecule is highly affected by the phospholipid acyl chain length, and within a series of saturated phosphatidylcholines and the best 'match' appears to be achieved with acyl chains having 14 to 17 carbons [5]. Studies on cholesterol/phospholipid model membranes have clearly shown the existence of a heterogeneous distribution of lipids in the plane of the mem-

Abbreviations: PC, phosphatidylcholine; di-10-PC, didecanoylphosphatidylcholine; di-12-PC, didodecanoylphosphatidylcholine; di-14-PC, ditetradecanoylphosphatidylcholine; di-15-PC, dipentadecanoylphosphatidylcholine; di-16-PC, dihexadecanoylphosphatidylcholine; NBD-cholesterol, 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3-ol.

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brane, suggesting that cholesterol specifically interacts with a phospholipid molecules to create lateral domains [6–8]. Consequently, at certain lateral surface pressures and compositions, laterally condensed phases (cholesterol-rich) coexist with an expanded phase (phospholipid-rich). This lateral heterogeneity is thought to arise from competing molecular interactions among the membrane constituents, these being mainly attractive van der Waals forces between adjacent hydrophobic molecular segments, and repulsive electrostatic forces between the polar moieties of the compounds [9–11].

The aim of this study was to examine lateral domain heterogeneity in phosphatidylcholine mixed monolayers at the air/water interface using monolayer fluorescence microscopy, and to correlate the heterogeneity with cholesterol concentration and phospholipid acyl chain length.

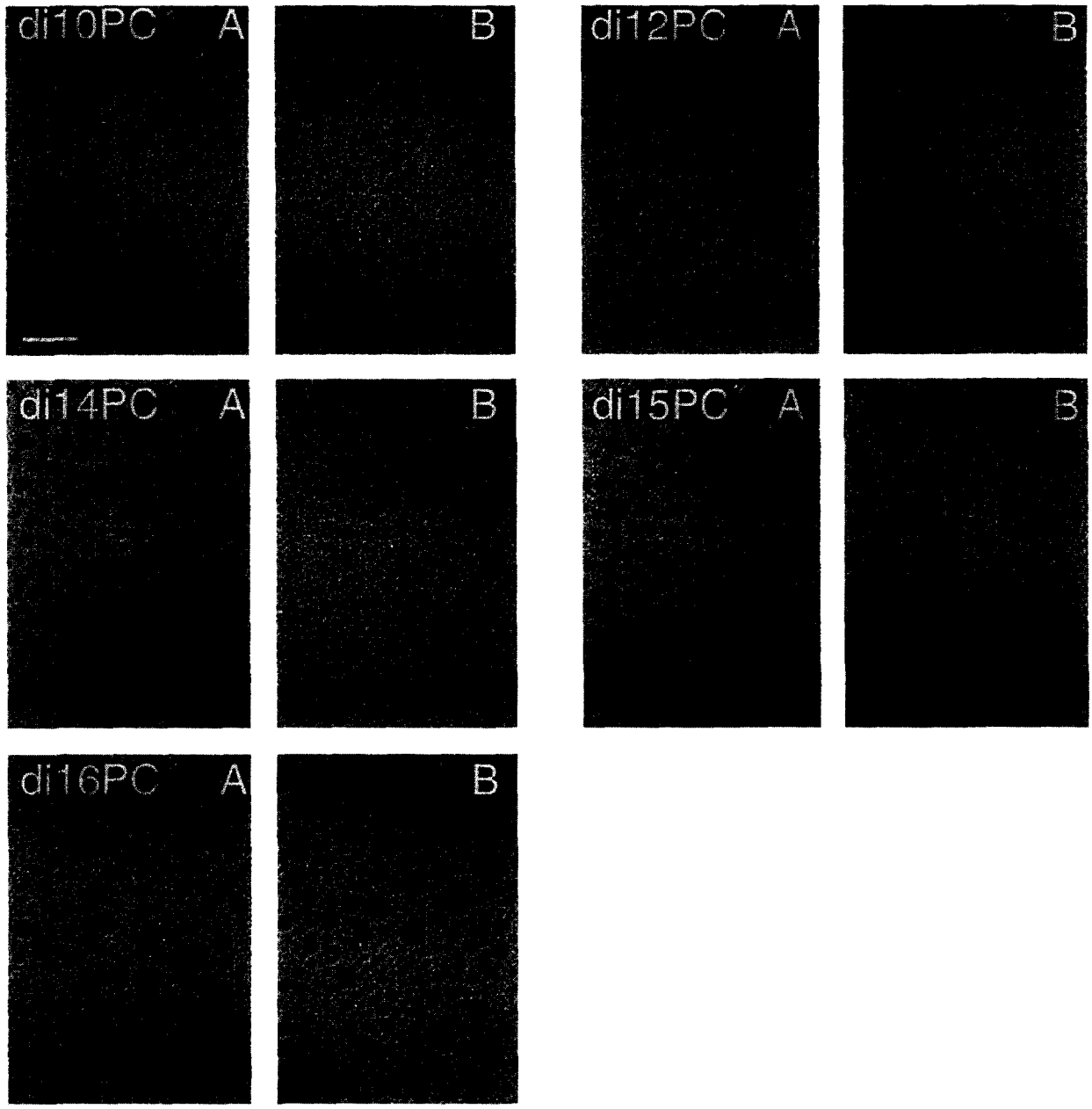

Fig. 1. Lateral domains in mixed monolayers containing 20 mol% cholesterol in different phosphatidylcholines. 30 nmol of lipids were spread on the clean water surface, and the formed monolayer was compressed (3.4 $Å^2$/molecule, min) to a lateral surface pressure that was about 2 mN/m below the characteristic phase transformation pressure for that particular monolayer (di10PC was documented at 17 mN/m, di12PC at 11 mN/m, di14PC at 7 mN/m, di15PC at 4 mN/m, and di16PC at 1 mN/m). Panels A show the domain properties immediately after the initial compression. The monolayers were then compressed to a lateral surface pressure which was 10 mN/m above the phase transformation pressure, after which they were allowed to expand back to the lateral surface pressure 2 mN/m below the phase transformation pressure. The domain properties after the compression/expansion cycle are shown in panels B (panels A and B were documented at the same lateral surface pressure). The scale bar represents 50 μm.

2. Materials and methods

2.1. Materials

Cholesterol and the phospholipids were obtained from Sigma (St. Louis). They were pure by thin-layer chromatography when used. When necessary, lipids were purified prior to use by BondElut NH_2 columns [12]. NBD-cholesterol was purchased from Molecular Probes (Oregon, USA). The water used as subphase was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system, to yield a product with a resistivity better than 18 MΩ/cm.

2.2. Formation of monolayers at the air / water interface

The equipment used for the visualization of monolayers consisted of a KSV Minisystems surface barostat which could be mounted on the stage of an Olympus IMT-2 inverted epifluorescence microscope. The barostat performed symmetric compression of the monolayer (i.e., with two barriers from each end of the trough), and the trough (24 111 mm^2 area) was equipped with a 50 mm diameter quartz window in the center, which allowed for excitation and observation of the monolayer fluorophores. 30 nmol of lipids were spread from a hexane/2-propanol stock solution on pure water to form mixed monolayers at 22°C. The available molecular area at film spreading was 133.5 $Å^2$. The monolayers contained either 20, 25, or 33 mol% cholesterol with different phosphatidylcholines, and 0.5 mol% NBD-cholesterol as a fluorescent probe. After application, the monolayers were compressed symmetrically at a speed of 3.4 $Å^2$/molecule, min to a lateral surface pressure which was 2 mN/m below the phase transformation pressure (indicated in the results section for each monolayer composition and type). At this lateral compression, the monolayers were observed with epifluorescence microscopy, and documented using a Hitachi video camera connected to a DT3851 digitizing board (Data Translation, Marlboro, MA). The monolayers were then compressed to a surface pressure which was about 10 mN/m above the phase transformation pressure of the monolayer. After this compression, the monolayer was allowed to expand to a lateral surface pressure which again was 2 mN/m below the phase transformation pressure. A new documentation of the monolayer lateral domains at this surface pressure was performed. The quantification of the average size of the lateral domains was performed on the digitized images using Image Pro plus (v. 1.2) or Global Lab Image (v. 2.2) software.

3. Results

Monolayers containing three different concentrations of cholesterol (20, 25, and 33 mol%) were prepared with phosphatidylcholines having different-length, symmetric, and saturated acyl chains (di10PC, di12PC, di14PC, di15PC, and di16PC). The fluorescent probe, NBD-cholesterol, was present at 0.5 mol%. This probe is known to partition preferentially into liquid-expanded domains (bright fluorescence), whereas it is excluded from more condensed domains (giving these a darker appearance) [8]. Since domain stability in mixed monolayers is an apparent function of the lateral surface pressure (the domain line boundary dissipates at a specific surface pressure [6,7,10]),

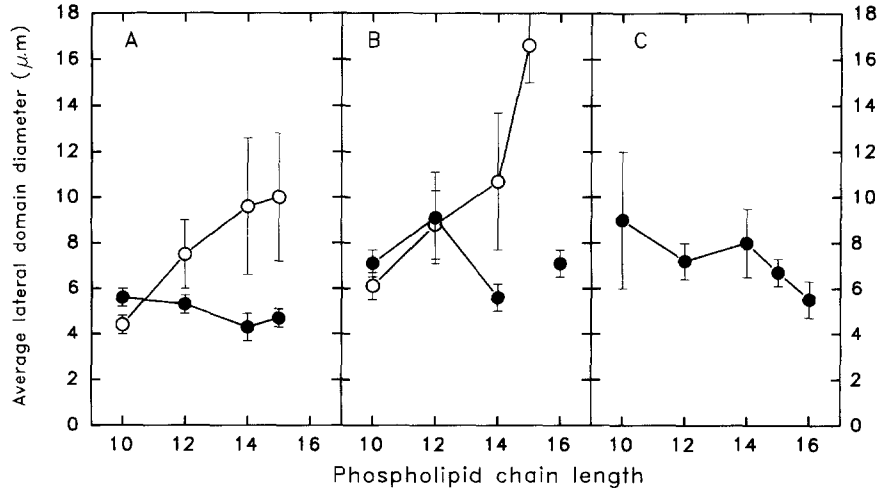

Fig. 2. Average lateral domain size in mixed cholesterol/phosphatidylcholine monolayers. The average size (diameter) of the circular domains was determined from four different micrographs of each monolayer type, using Image Pro plus image analysis software. Values given are averages ± S.D. Panel A shows domains in mixed monolayers containing 20 mol% cholesterol, panel B is with 25 mol% cholesterol, and panel C with 33 mol% cholesterol. Open symbols are for domains observed before the phase transformation pressure (corresponding to domains seen in panels A of Figs. 1 and 5), and solid symbols are for domains observed after a compression/expansion cycle (corresponding to domains seen in panels B of Figs. 1, 5, and 6). Panel A and B shows sizes for liquid-condensed domains (except di16PC in panel B which is for liquid-expanded domains). Panel C shows sizes for liquid-expanded domains.

lateral domain properties were determined during initial compression, and during monolayer expansion (after a compression/expansion cycle which took the monolayer well above the phase transformation pressure).

3.1. Monolayers with 20 mol% cholesterol

Lateral liquid-condensed domains against a liquid-expanded phase were formed with all phosphatidylcholines used (di10PC-di16PC; Fig. 1). The liquid-condensed domains in a cholesterol/phosphatidylcholine system with more than or about 10 mol% sterol are known to be cholesterol-rich, since their number increase with increasing cholesterol concentration [13]. Similar sterol-rich (laterally condensed) domains were not formed in 4-cholesten-3-one/phosphatidylcholine mixed monolayers [14], further demonstrating that the liquid-condensed domains seen in cholesterol/phosphatidylcholine monolayers resulted from a specific interaction between cholesterol and the phospholipid. The results of this study show that the liquid-condensed domain size (diameter) during initial compression (Fig. 1, panels A) appeared to increase with increasing phosphatidylcholine acyl chain length (Fig. 2A). The size distribution of the liquid-condensed domains during initial compression appeared to be rather homogenous for all phosphatidylcholines except di16PC (Fig. 1, panels A). The liquid-condensed domains of panels A in Fig. 1 were documented at a lateral surface pressure which was about 2 mN/m below the phase transformation pressure (see Fig. 3). When the mixed monolayers were compressed further, domain line boundaries dissipated at the phase transformation pressure. This characteristic phase transformation pressure was highest with short chain phosphatidylcholines and lowest with longer chain phosphatidylcholine

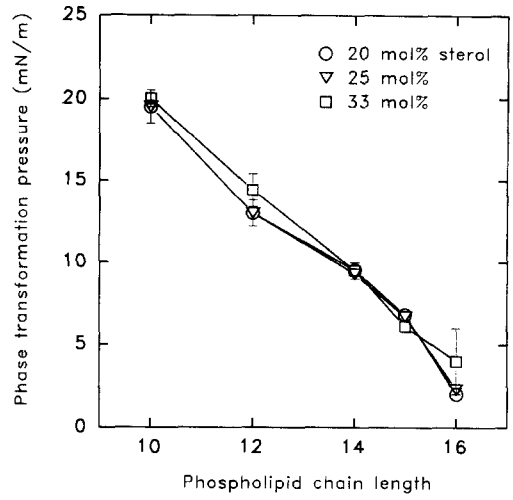

Fig. 3. Phase transformation pressures in mixed cholesterol/phosphatidylcholine monolayers. The lateral surface pressure at which the domain line boundaries begun to dissipate were recorded for each cholesterol concentrations tested (20, 25, and 33 mol%) with the different phosphatidylcholines. Each value is the mean of four different experiments (±S.D.).

(Fig. 3). The monolayers were then compressed to a lateral surface pressure which was 10 mN/m above the phase transformation pressure, and expanded to a lateral surface pressure 2 mN/m below the phase transformation pressure. The domains formed during this monolayer expansion apparently represent thermodynamically more stable structures [6]. Panels B of Fig. 1 show the liquid-condensed domains of the mixed monolayers which have gone through a compression/expansion cycle. The domains formed during expansion of the monolayers were liquid-condensed, against a background of the liquid-expanded

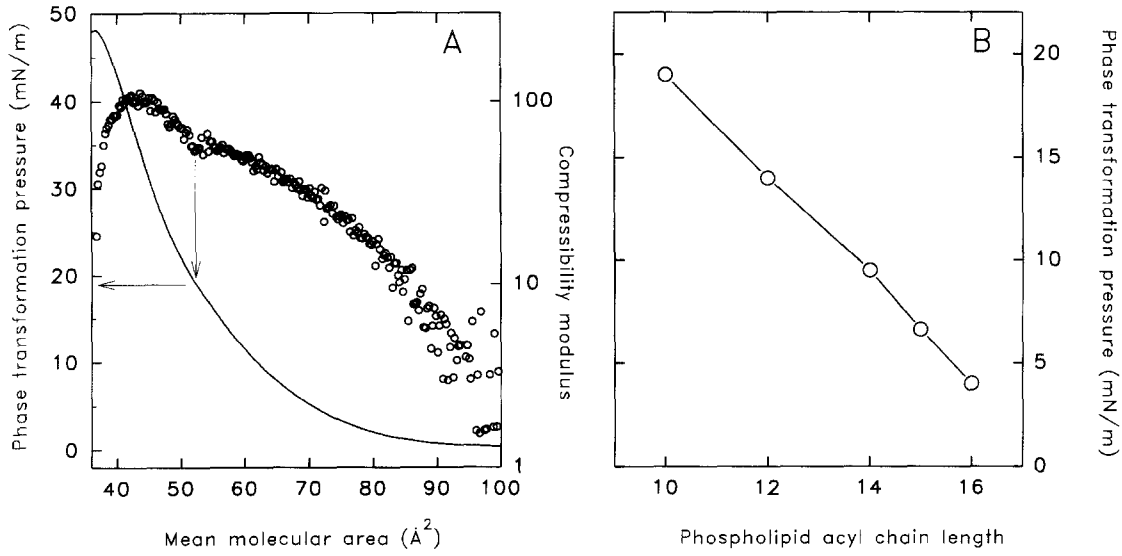

Fig. 4. Analysis of force–area isotherms of mixed cholesterol/phosphatidylcholine monolayers (20:80 mol ratio). The inverse compressibility function of the force-area isotherm was plotted against the mean molecular area, and the discontinuity of this function (phase transformation) was correlated with the corresponding lateral surface pressure (panel A shows the data for a cholesterol/di10PC mixed monolayer)). The so derived phase transformation pressure was plotted as a function of phosphatidylcholine acyl chain length, as given in panel B (for all mixed phosphatidylcholine monolayers).

phase. It can further be seen that the domains were smaller in size after the compression/expansion cycle than they were during the initial compression (Figs. 1 and 2). The exceptions were di10PC and di16PC, which yielded similar liquid-condensed domains with cholesterol during compression and expansion.

3.2. Derivation of the phase transformation pressure from the force–area isotherm

The surface pressure at which the phase transformation occurs can also be obtained from analysis of force–area isotherms [10,15]. If one plots the inverse of the compressibility ($= -A\Delta\pi/\Delta A$; where A is the molecular area, and $\Delta\pi$ the change in surface pressure per change in molecular area) against the mean molecular area, and compares this isotherm with the force–area isotherm, a situation shown in Fig. 4A, can be seen. The break in the otherwise symmetric inverse compressibility function shows where the transition occurs. From this break, one can obtain the mean molecular area and also the lateral surface pressure for this change. When all phosphatidylcholine monolayers (containing 20 mol% cholesterol) were analyzed in this way, the phase transformation pressures shown in Fig. 4B were obtained. It is evident that the phase transformation pressures obtained from monolayers

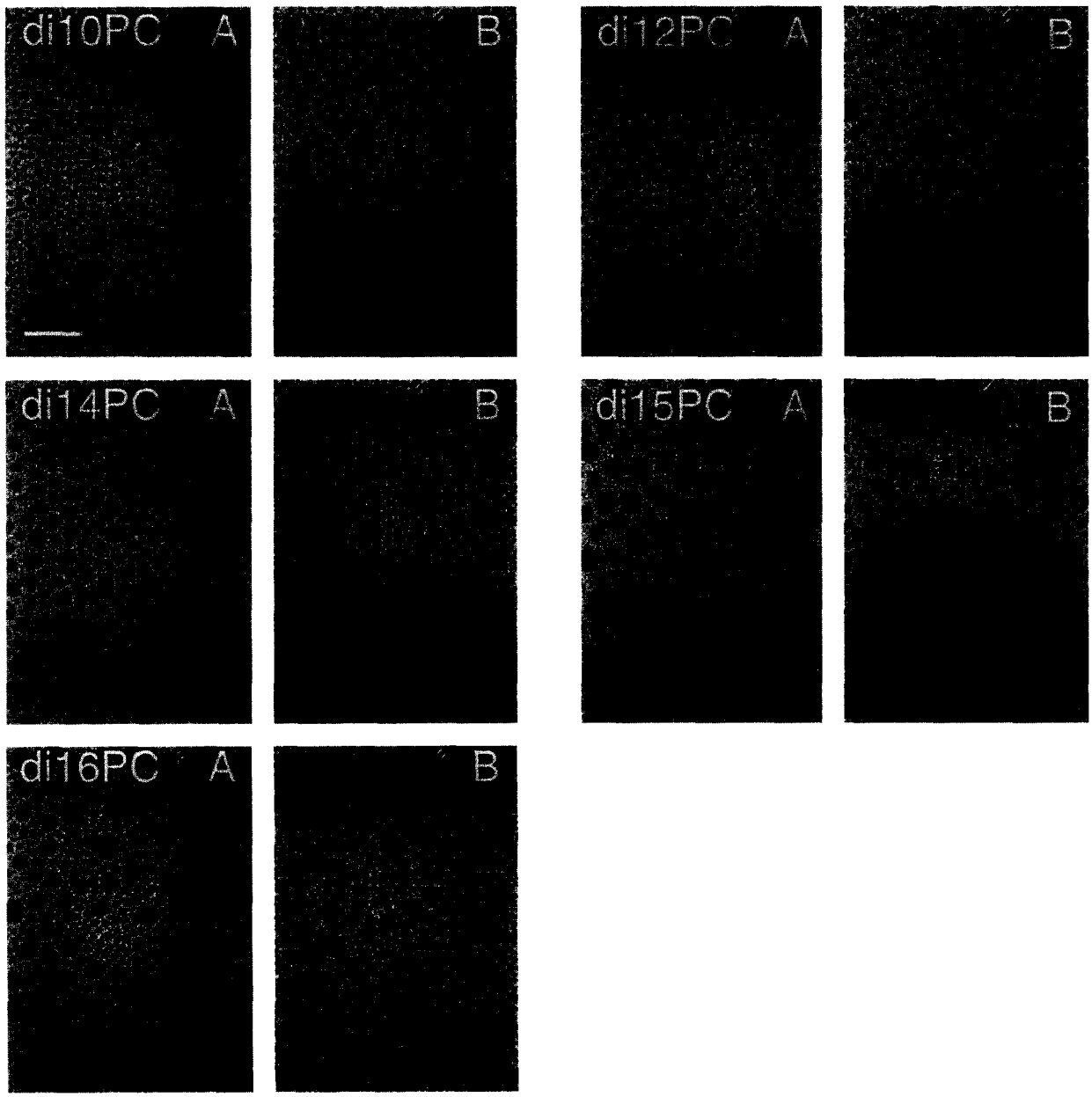

Fig. 5. Lateral domains in mixed monolayers containing 25 mol% cholesterol in different phosphatidylcholines. Experimental conditions are similar to those described for in the legend to Fig. 1.

without fluorescent probe were identical to the transformation pressures obtained from microscopic analysis of comparable mixed monolayers containing 0.5 mol% NBD-cholesterol (Fig. 3). Therefore, one can conclude that the presence of NBD-cholesterol did not measurably affect the phase transformation pressure.

3.3. Monolayers with 25 mol% cholesterol

With 25 mol% cholesterol in the mixed monolayers, similar liquid-condensed domains were formed as was seen with the 20 mol% system (Fig. 5). The liquid-condensed domains were fairly homogenous in size, and the size of the domains again increased with increasing phosphatidylcholine acyl chain length (Fig. 2B). The exception was again di16PC which had a heterogeneous domain size distribution (Fig. 5). The phase transformation pressure in these monolayers were similar to those observed in the mol% system (Figs. 3 and 4), suggesting that the phase transformation pressure was not markedly affected by the cholesterol content of the mixed monolayers. The domains formed in monolayers that underwent a compression/expansion cycle are shown in panels B of Fig. 5. Di10PC and di12PC gave liquid-condensed domains which were similar before and after the compression/expansion cycle, whereas the liquid-condensed domains in di14PC monolayers were markedly reduced in size after the expansion as compared to before it (Figs. 5 and 2B). With di15PC a

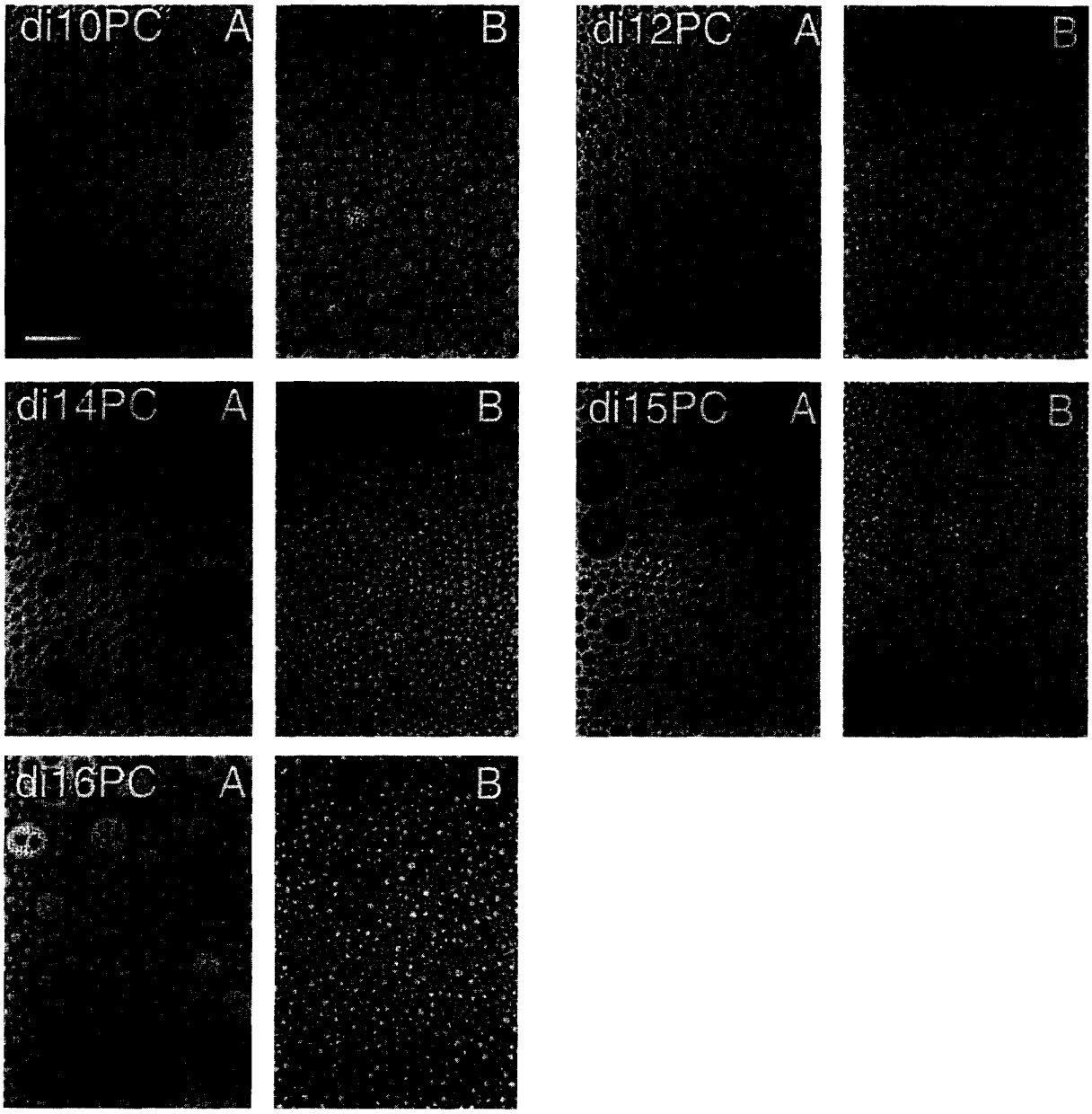

Fig. 6. Lateral domains in mixed monolayers containing 33 mol% cholesterol in different phosphatidylcholines. Experimental conditions are similar to those described for in the legend to Fig. 1.

partial fusion of liquid-condensed domains was seen after the compression/expansion cycle, whereas the fusion of liquid-condensed domains was almost complete with di16PC monolayers (Fig. 5). The fusion of the liquid-condensed domains in di16PC monolayers gave these an inverted appearance as compared to the situation before the compression/expansion cycle (Fig. 5, di16PC panel A versus B).

3.4. Monolayers with 33 mol% cholesterol

The lateral domains formed in mixed monolayers having 33 mol% cholesterol were no longer homogenous in size distribution during the initial compression (Fig. 6, panels A). The liquid-condensed domains, seen against a liquid-expanded phase, were rather similar for phosphatidylcholine having acyl chains between 10 and 15 carbons in length. However, with di16PC, the monolayer showed liquid-expanded domains against a liquid-condensed phase. Since this was observed during initial compression, the liquid-condensed domains had coalesced already before the phase transformation pressure. The phase transformation pressure in monolayers with 33 mol% cholesterol was rather similar to the pressures observed for monolayers with lower cholesterol content (Fig. 3). The lateral domains formed in monolayers that underwent a compression/expansion cycle were liquid-expanded against a background of a liquid-condensed phase (i.e., inverted with respect to the situation during initial compression, except with di16PC; panels B or Fig. 6), indicating that liquid-condensed domains were triggered to coalesce when the monolayer passed through the phase transformation pressure. The size of the liquid-expanded domains formed after a compression/expansion cycle were larger with short chain phosphatidylcholines and smaller with longer chain phosphatidylcholines (panels B or Fig. 6, and Fig. 2C). The size distribution of the liquid-expanded domains were quite homogenous (Fig. 6).

4. Discussion

The formation of laterally condensed domains (cholesterol-rich) in an expanded phase (phospholipid-rich) is the result of molecular association between cholesterol and the phospholipids, leading to the formation of large (with respect to the size of the molecules) areas with a more ordered lipid packing than is found in the bulk surrounding phase. The fractional area of this laterally condensed, cholesterol-rich phase increases sigmoidally with increasing cholesterol concentration [13]. The circular shapes of the domains (both liquid-condensed domains against a bulk liquid-expanded phase, and liquid-expanded domains against a bulk liquid-condensed domain) clearly indicate that these are liquid-like and have a high line tension [16].

This study has shown that the phosphatidylcholine acyl chain length has a marked effect on the properties of lateral condensed (cholesterol-rich) domains in mixed cholesterol/phosphatidylcholine monolayers. Several acyl-chain-dependent trends are evident in the properties of the domains. Both the size of the liquid-condensed domains (at 20 and 25 mol% cholesterol during initial compression), the size of the liquid-expanded domains (at 33 mol% cholesterol after a compression/expansion cycle), the tendency of liquid-condensed domains to coalesce, and the phase transformation pressure (the pressure at which the monolayer is transformed from a two-phase coexistence to an apparent one phase system), all appeared to markedly depend on the acyl chain length of the phosphatidylcholines. These four monolayer domain characteristics will be addressed in turn.

First, the size of the liquid-condensed domains during initial compression was a function of the phosphatidylcholine acyl chain length, with liquid-condensed domains being larger with acyl-chains becoming longer. This phenomenon is hardly of any real significance, since the domain sizes during initial compression appears not to be representative of equilibrium domain sizes. This is indicated by the finding that domain sizes decrease significantly after the monolayer has experienced a compression/expansion cycle, after which the reformed domains are much smaller, and more homogenous in size. In addition, and most importantly, there appears no longer to be an acyl chain dependence of the liquid-condensed domain size. McConnell and coworkers have previously also reported that liquid-condensed domains become smaller and more homogenous in their size distribution after the mixed cholesterol/phosphatidylcholine monolayer is first compressed beyond the phase transformation pressure, and then allowed to expand to a low surface pressure at which liquid-condensed domains again reform [6,7].

Domain shapes are markedly affected by factors such as line tension (gives circular domains), the alignment of molecular dipole functions (favors elongated and branched shapes), the experimental temperature, monolayer impurities, and compression rates [11,17–22]. In our model system with phosphatidylcholine having short or intermediate length acyl chains, their physical state at the temperature of the experiments (22°C) is fluid, especially in the presence of moderate amounts of cholesterol. Therefore, line tension is expected to be the dominant effector of domain shape. Due to the fluid nature of the mixed monolayers, circular domain shapes are also favored by the entropic contribution to the system, leading to randomization of the dipole alignment and to a minimization of the tendency of dipole repulsion to favor domain shape elongation and branching [18]. The relative strength of intermolecular association (between cholesterol and a phosphatidylcholine), and consequently the formation of long-range order (i.e., domains), is expected to be dependent on the possibility for van der Waals forces to form. More van der

Waals forces are expected to form between cholesterol and di16PC than between cholesterol and di10PC [5], and therefore it is conceivable that the large domains formed (during initial compression) with longer chain phosphatidylcholine as compared to the shorter chain analogues were in part due to the effect of van der Waals forces.

Secondly, the size of the liquid-expanded domains (phospholipid-rich) in the liquid-condensed phase of mixed monolayers (with 33 mol% cholesterol) which had experienced a compression/expansion cycle also appeared to be a function of the phosphatidylcholine acyl chain length. Liquid-expanded domains were larger with shorter chains and smaller with longer chains. Since these structures are though to be close to equilibrium shapes (due to the compression/expansion cycle), they probably truly reflect the effect of molecular structure on domain shape. Since phosphatidylcholines with shorter acyl chains have a more expanded force–area isotherm as compared with longer chain analogues [4,5], and consequently have a larger molecular area requirement, this molecular property most likely contribute to the size of the liquid-expanded domains in the mixed monolayers.

Third, the tendency of liquid-condensed domains to coalesce was clearly dependent both on the phosphatidylcholine acyl chain length and on the cholesterol content of the mixed monolayers. During initial compression (non-equilibrium shapes), liquid-condensed domains were partly coalesced in di16PC monolayers at 20 and 25 mol% cholesterol (indicated by the heterogeneous size distribution of the liquid-condensed domains), whereas at 33 mol% cholesterol di10PC to di15PC mixed monolayers had partly and di16PC monolayers completely coalesced liquid-condensed domains. After the compression/expansion cycle, there was partial or complete fusion of liquid-condensed domains at 25 mol% cholesterol with di15PC and di16PC, respectively, and at 33 mol% cholesterol, all phosphatidylcholines showed fusion of the liquid-condensed domains. It is clear that the compression/expansion cycle could trigger the fusion of liquid-condensed domains in such monolayers where the fused liquid-condensed domains appeared to be thermodynamically more favorable. Since both the concentration of cholesterol and the phosphatidylcholine acyl chain length affected the tendency of liquid-condensed domains to fuse, one can argue that more (cholesterol concentration) and stronger (more van der Waals forces with no longer acyl chains) intermolecular associations resulted, which in turn favored the formation of a very large cholesterol-rich domain. The fusion of cholesterol-rich domains observed in these monolayers appears to be analogous to a similar fusion of cholesterol-rich domains calculated to take place in bilayer membranes of di16PC having a cholesterol concentration at or above 20 mol% [23]. A fusion of liquid-condensed domains similar to that observed in di16PC monolayers was also seen in *N*-palmitoylsphingomyelin mixed monolayers [13], suggesting that the propensity for fusion of liquid-condensed domains is related to the phospholipid acyl chain configuration.

Finally, the characteristic surface pressure at which the domain line boundary dissipated, bringing the monolayer from a two-phase coexistence into an apparent one-phase system (phase transformation pressure) varied markedly with the phosphatidylcholine acyl chain length (higher phase transformation pressure with shorter chains and vice versa). Theoretical considerations have implied that laterally condensed domains in cholesterol/phosphatidylcholine mixed monolayers are stabilized by van der Waals attractive forces, and destabilized by repulsive electrostatic interactions [9,10]. Immediately below the phase transformation pressure, both the attractive and the repulsive forces are balanced, but as the monolayer is further compressed, electrostatic repulsive interactions begin to dominate over the attractive forces, and as a consequence, the domain wall energy disappears. This eventually leads to the dissipation of the domain line boundary. In our case with different length phosphatidylcholines, the permanent dipole of the polar function of the phosphatidylcholine is similar with all phosphatidylcholine analogues (although the dipole orientation may differ slightly from one analogue to the other due to packing differences). However, the strength of interaction between cholesterol and the phosphatidylcholines differ. It is plausible to assume that the high phase transformation pressure observed in mixed monolayers with di10PC resulted from a weak electrostatic repulsion, which in turn was due to the relatively long distance between polar head groups in these very expanded monolayers (cf. [5]). As the phosphatidylcholine acyl chain length increased, the attractive van der Waals forces increased, but so did the repulsive forces, since the packing density increased (and hence the distance between repulsive dipoles decreased). Consequently, with increasing the phosphatidylcholine acyl chain length, the electrostatic repulsion appeared to be stronger than the attractive van der Waals forces, and the phase transformation was observed as lower lateral surface pressures. The phase transformation pressure was not affected by the presence of an impurity in the membrane (0.5 mol% NBD-cholesterol), since a similar phase transformation pressure was seen in mixed monolayers without the fluorescent probe. This latter phase transformation pressure was deduced from analysis of the force–area isotherms [10,15], and agreed remarkably well with results obtained by visual documentation of the phase transformation pressure.

These studies have demonstrated the effect of varying the phosphatidylcholine acyl chain length on the formation of laterally segregated sterol-rich domains in mixed monolayers at the air/water interface. From these results it can be assumed that van der Waals forces acting between the acyl chains of the phosphatidylcholines, and between acyl chain segments and the sterol molecule are an important contributing determinant of the lateral distribution of cholesterol in phospholipid membranes. Even tough the

lateral sterol-rich domains are not visible at the resolution of the light microscope when the membranes are compressed above the phase transformation pressure, a heterogeneous distribution of cholesterol in bilayer membranes is still evident in many different types of experiments [24–26].

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