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Interactions of Cyclosporin A with Phospholipid Membranes: Effect of Cholesterol

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ABSTRACT

Cyclosporin A (CsA) is a highly hydrophobic drug used to prevent graft rejection after organ transplantation. Interactions of CsA with phosphatidylcholine as well as with binary mixtures containing phosphatidylcholine and cholesterol were investigated by measuring the penetration of CsA into lipid monolayers at an air/water interface, by differential scanning calorimetry, and by imaging with fluorescence microscopy the effects of CsA on the lateral distribution of a fluorescent probe, 1-palmitoyl-2-(*N*-4-nitrobenz-2-oxa-1,3-diazol)aminocaproyl-phosphocholine, in monolayers. Film penetration studies revealed the association of CsA with lipids to be a biphasic process. Cholesterol diminished the intercalation of CsA into the monolayer at surface pressures of $>$19 mN/m. CsA broadened the main transition of dimyristoylphosphatidylcholine (DMPC)/β-cholesterol (10:1, mol/mol) multilamellar vesicles. The behavior of the transition enthalpy was more complex; the behavior of DMPC/β-cholesterol multilamellar vesicles in the X_{CsA} of 0 to 0.1 showed at most ratios a increase, but several well distinct dips were observed. The results are interpreted in terms of regular structures in tertiary alloy. Influence of CsA on lateral organization could be verified for lipid domains observed by fluorescence microscopy of lipid monolayers. More specifically, CsA altered the distribution of 1-palmitoyl-2-(*N*-4-nitrobenz-2-oxa-1,3-diazol)aminocaproyl-phosphocholine in a dipalmitoylphosphatidylcholine film and in DPPC/β-cholesterol (88:10, mol/mol) mixtures in a manner that suggests that CsA partitions into the boundaries between fluid and gel domains. To our knowledge, this constitutes the first demonstration of a change in lipid domain morphology to be induced by a drug molecule.

Cyclosporin A (CsA) is a key element in the struggle against rejection in organ transplantation; its introduction into the immunosuppression in 1980s resulted in a major improvement in graft survival (Cohen et al., 1984). This fungal protein is composed of a 11-residue cyclic peptide containing two uncommon amino acids: (4*R*)-4-[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine and L-α-aminobutyric acid, as well as seven peptide bond *N*-methylated residues. Two conformations have been demonstrated for CsA: one for the unbound (free) peptide crystallized form in an organic solvent and the other for CsA-immunophilin complex or CsA bound to the phosphatase calcineurin (O′Donohue et al., 1995). The binding of CsA to calcineurin is thought to be an important step in its mechanism of action, leading to the decreased synthesis of interleukin-2 (Hemar and Dautry-Varsat, 1990). The clinical use of CsA is limited by its nephrotoxicity, with the molecular level mechanisms of this side effect remaining unresolved (Kopp and Klotman, 1990). CsA has been recently reported to block the opening of mitochondrial permeability transition pore and to interfere with the induction of apoptosis (Bernardi, 1996). Contrasting with these findings, CsA has been shown to also induce apoptosis in some cell lines (Kitagaki et al., 1997).

Due to its profound hydrophobicity, CsA avidly partitions into membranes. CsA has been shown to abolish the pretransition for saturated phospholipids and to decrease both their main transition temperature and enthalpy (O′Leary et al., 1986; Wiedmann et al., 1990). CsA decreases acyl chain order at temperatures below main phase transition, whereas increased order at temperatures above the transition is evident (Wiedmann et al., 1990). CsA increases the lamellar-to-hexagonal phase transition temperature of dielaidoylphosphoethanolamine at low drug-to-lipid molar ratios, whereas a decrease is seen at higher contents of CsA (Epand et al., 1987). CsA also inhibits membrane fusion (Epand et al., 1987).

Coupling between membrane organization and function is emphasized in the modern view of membrane biology, and

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ABBREVIATIONS: CsA, cyclosporin A; α-cholesterol, 5-cholesten-3α-ol; β-cholesterol, 5-cholesten-3β-ol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMSO, dimethylsulfoxide; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; eggPC, egg yolk phosphatidylcholine; ΔH, main phase transition enthalpy; ΔH_0, the main phase transition enthalpy of the DMPC/β-cholesterol (10:1, mol/mol) MLVs; NBD-PC, 1-palmitoyl-2-(*N*-4-nitrobenz-2-oxa-1,3-diazol)aminocaproyl-phosphocholine; MLV, multilamellar vesicles; π, surface pressure; $\Delta\pi$, change in surface pressure; π_0, initial surface pressure; π_c, critical lipid lateral pressure; T_m, temperature of main phase transition; X, mole fraction of the indicated substance.

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several molecular level processes generating dynamic order have by now been established in model membranes. Both peripheral and integral membrane proteins, as well as metabolites, ions and drugs, and changes in the temperature and degree of phospholipid hydration, for instance, can alter membrane organization (Kinnunen, 1991; Mouritsen and Kinnunen, 1996). On the other hand, changes in membrane organization can also influence the association of different ligands to the membrane, as shown for doxorubicin (Söderlund et al., unpublished observations), cytochrome *c* (Mustonen et al., 1987), and histone H1 (Rytömaa and Kinnunen, 1996). Accordingly, there is a reciprocal interplay between membrane organization and binding of ligands to membranes.

Changes in membrane organization have been suggested to be induced by several drugs, such as tacrine, which is used in the treatment of Alzheimer's disease (Lehtonen et al., 1996); the anticancer drug doxorubicin (Goormaghtigh et al., 1982), local anesthetic agents (de-Paula and Schreier, 1996); and the antifungal drug amphotericin B (Lance et al., 1996). A recent approach to molecular-level mechanisms of action (or side effects) of drugs is to elucidate their interactions with membranes and effects on membrane organization in relation to their membrane-involving functions. Due to the high lipid solubility of CsA, it was of interest to study the interactions of this compound with phospholipid membranes. Likewise, because some of the effects of CsA reported so far resemble those exerted by cholesterol, we also investigated the influence of this sterol on the interaction of CsA with lipid membranes.

Experimental Procedures

Materials. HEPES and EDTA were purchased from Sigma (St. Louis, MO), and dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), egg yolk phosphatidylcholine (eggPC; molecular weight, ~750), 5-cholesten-3α-ol (α-cholesterol), and 5-cholesten-3β-ol (β-cholesterol) were obtained from Sigma, and 1-palmitoyl-2-(*N*-4-nitrobenz-2-oxa-1,3-diazol)aminocaproyl-*sn*-glycero-3-phosphocholine (NBD-PC) was obtained from Avanti Polar Lipids (Alabaster, AL). CsA was a kind gift from Novartis (Basel, Switzerland). The purity of lipids was checked by thin-layer chromatography on a silicic acid-coated plates (Merck) using chloroform/methanol/water (65:25:4, v/v) as a solvent system. Examination of plates after iodine staining or fluorescence illumination revealed no impurities. To assess the surface chemical purity of DPPC, eggPC, and β-cholesterol, their pressure/area (π-A) isotherms were recorded using a computer-controlled Langmuir film balance (μTroughS; Kibron Inc., Helsinki, Finland). The isotherms obtained were in keeping with those in the literature. The concentrations of lipid and drug solutions were determined gravimetrically using a high precision electrobalance (Cahn Instruments, Inc., Cerritos, CA) or spectrophotometrically using ϵ = 19,000 M^{-1} at 465 nm for NBD-PC. Molecular weights of 752 and 724 were used for DPPC and DMPC, respectively, corresponding to their monohydrates.

Penetration of CSA into Lipid Monolayers. Penetration of CsA into monomolecular lipid films was measured using magnetically stirred circular wells (surface area, 31 cm^2; volume, 50 ml) drilled in Teflon. Surface pressure (π) was monitored with a platinum Wilhelmy plate attached to a microbalance connected to a 486 PC via a DT01-EZ data acquisition board. Aqueous phase was 5 mM HEPES and 0.1 mM EDTA (pH 7.4). Lipids were spread on the air-water interface in chloroform (approximately 1 mg/ml) and were allowed to equilibrate for 20 min so as to reach different initial surface pressures (π_0) before the addition of CsA (5 μl, 2 mg/ml in DMSO) into the subphase. The increment in π after the addition of CsA was complete in approximately 20 min, and the difference between the initial surface pressure (π_0) and the value observed after the intercalation of the drug into the film was taken as $\Delta\pi$. All measurements were performed at ambient temperature (~+24°C). The data are represented as $\Delta\pi$ versus π_0, thus revealing the decrement in the intercalation of CsA into lipid monolayer on increasing lateral packing density of the film. These graphs also yield the critical surface pressure π_c (i.e., lipid lateral packing density preventing the penetration of the drug into film).

Differential Scanning Calorimetry. Phospholipids, cholesterol, and CsA were codissolved in chloroform to obtain the desired lipid-to-drug ratios. Solvent was first evaporated under a stream of nitrogen, and the dry residues then were maintained under reduced pressure for a minimum of 2 h. Subsequently, samples were hydrated in a buffer (5 mM HEPES, 0.1 mM EDTA, pH 7.4) for 30 min at a temperature of approximately 10°C over the main transition to yield multilamellar vesicles (MLVs) at a total phospholipid concentration of 0.77 mM. Liposomes were maintained at +4°C overnight before recording heat capacity scans with a high-sensitivity differential scanning microcalorimeter (VP-DSC; Microcal Inc., Northampton, MA), operated at a heating rate of 0.5°C/min. All samples had the same thermal history. The calorimeter was connected to a Pentium PC, and data were analyzed using commercial software (Origin; Microcal Inc., Northampton, MA). Transition enthalpies are expressed as kilojoules per mole of phospholipid and were determined by intergration of the peaks, using the internal calibration of the instrument as a reference. The deviation from the baseline was taken as the beginning of the transition and the point of return to the baseline as the end of the transition. Data points represent the mean for triplicate analyses, and the error bars indicate S.D.

Fluorescence Microscopy of Lipid Monolayers. Effect of CsA on the lateral distribution of NBD-PC was studied using a Zeiss IM-35 inverted microscope combined with a computer-controlled monolayer apparatus (μTrough S). Total surface area of the trough is 120 cm^2, and the volume of the subphase is 25 ml. The trough was mounted on the microscope stage, and the quartz-glass window in the bottom of the trough was positioned over an extralong working distance 20× objective. A 450- to 490-nm bandpass filter was used for excitation, and a 520-nm long-pass filter was used for emission. Images were recorded with a Peltier-cooled 12-bit digital CCD camera (C4742–95, Hamamatsu, Japan) interfaced to a computer and operated by the software (HiPic 4.2.0a) provided by the manufacturer.

The indicated lipids and CsA were mixed in an organic solvent (hexane/isopropanol/water, 70:30:2.5, v/v) and subsequently applied on the air-water interface using a microsyringe. After an equilibration period of 10 min, the monolayer was compressed symmetrically at a rate of 1 Å^2/chain/min. After reaching the desired values for π, the compression was

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stopped, and the monolayer was allowed to settle for 10 min before recording of the image. During this equilibration period, a decrease (approximately 0.9–5.4 mN/m) in surface pressure was observed, with the magnitude of the decrement in π depending on the film composition as well as the pressure range. The decrease in π represents the reorganization and relaxation of the monolayer toward the free energy minimum after the compression. Likewise, although the solubility of CsA into water is very low, there could be some desorption of this compound into the subphase. Accordingly, it is essential to note that the patterns shown are unlikely to represent true equilibrium states. However, because identical compression rates and equilibration times were used in each experiment, the results thus obtained should be amenable for comparison. All measurements were performed at ambient temperature (~+24°C).

Results

Binding of CsA to Lipid Monolayers. Although the association of CsA with liposomes composed of neutral zwitterionic lipids is well established (O′Leary et al., 1986; Wiedmann et al., 1990), we are not aware of studies on the penetration of CsA into phospholipid monolayers as a function of their lateral packing density. Increment in surface pressure ($\Delta\pi$) as a function of time, after the addition of CsA into the subphase underneath a eggPC monolayer at an initial surface pressure (π_0) of 15.4 mN/m, is illustrated in Fig. 1. As expected from the hydrophobicity of CsA, the penetration of this peptide into the film was rapid. Similar measurements were subsequently made at varying values for π_0, and the data are illustrated as $\Delta\pi$ versus π_0 (Fig. 2A). Interestingly, the data readily reveal the dependence of the interaction of CsA with the monolayer as a function of π_0 to be biphasic. More specifically, at surface pressures below ≈19 mN/m, the slope of the $\Delta\pi$ versus π_0 indicates a limiting value π_c of 25 mN/m for the penetration of CsA into the film. However, penetration of CsA is clearly augmented for monolayers initially maintained at π_0 >19 mN/m, and for these films, a significantly higher limiting value of π_c ≈35 mN/m, prohibiting the penetration of CsA into the film, is measured.

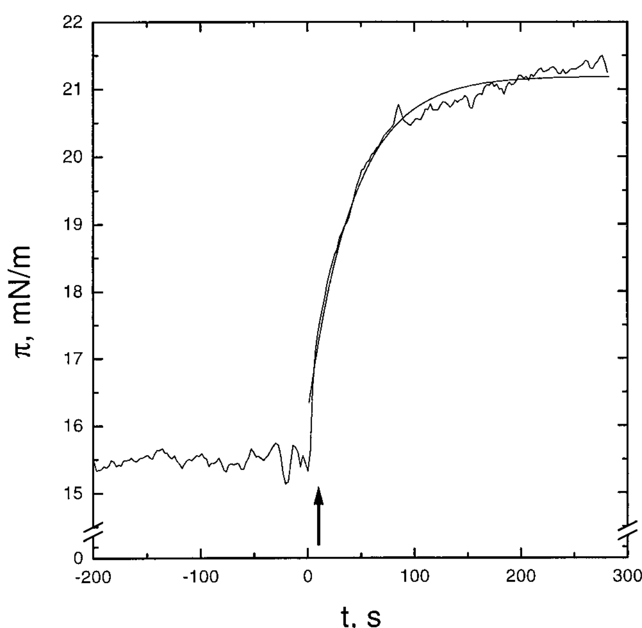

Fig. 1. Time course of the increase in surface pressure ($\Delta\pi$) for an eggPC monolayer over an aqueous subphase (5 mM HEPES, 0.1 mM EDTA, pH 7.4) after the addition of 10 μg of CsA (final concentration, 167 nM). Initial surface pressure (π_0) was 15.4 mN/m. The contents of the subphase were mixed by magnetic stirring. The experiment was carried out at ambient temperature (≈+24°C). The addition of CsA is marked by an arrow.

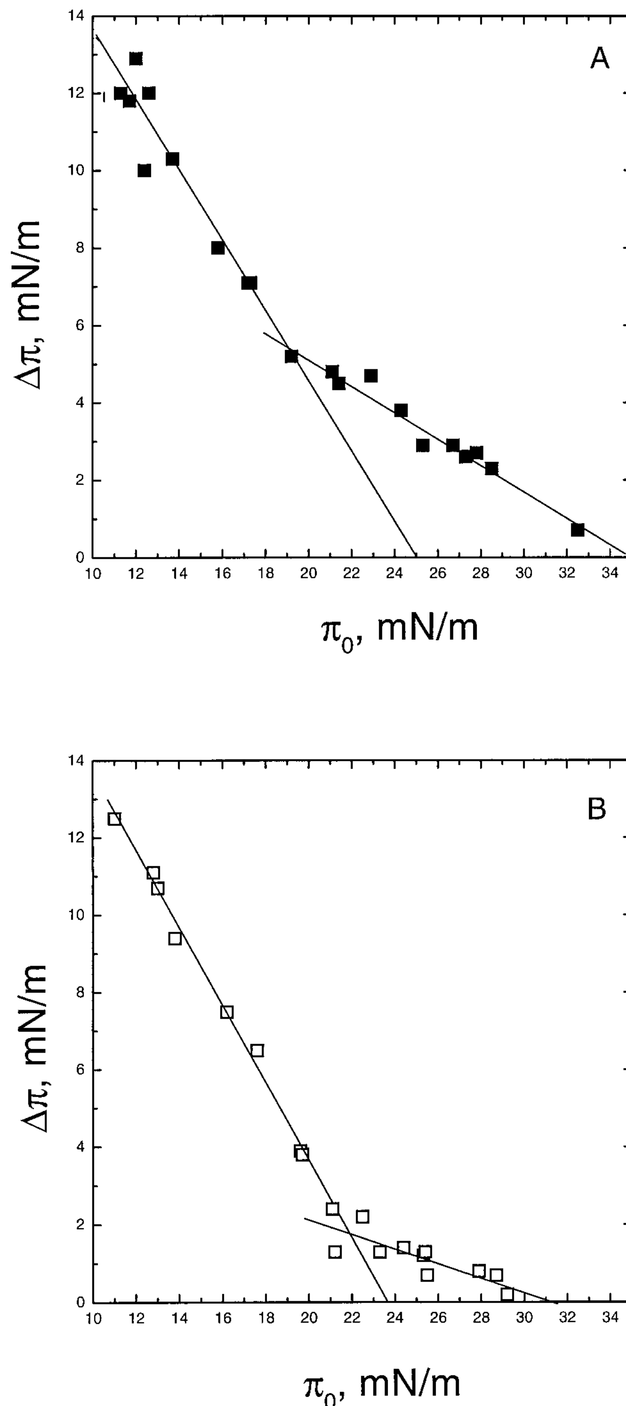

Fig. 2. Increase in surface pressure ($\Delta\pi$) due to the addition of CsA into the subphase and as a function of initial surface pressure (π_0). The monolayers used were eggPC (A) and eggPC/β-cholesterol (1:1, mol/mol; B). The lines drawn were calculated using least-squares linear fitting, with the following correlation coefficients for the different parts of the graphs: A, r = −0.96569 (π = 11.3–19.2) and r = −0.98175 (π = 19.2–32.5); B, r = −0.99737 (π = 11–21.1) and r = −0.83944 (π = 21.1–29.2). Conditions were as described in the legend for Fig. 1.

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The effects of CsA on the phospholipid (DPPC) acyl chain order below and above T_m (Wiedmann et al., 1990) resemble those observed for cholesterol (e.g., Ipsen et al., 1987). Accordingly, to explore possible interference by cholesterol on the lipid association of CsA, experiments on the penetration of this drug also were performed for eggPC/β-cholesterol (1:1, mol/mol) monolayers (Fig. 2). At π_0 from approximately 10 to 19 mN/m, the addition of CsA induces similar changes in the surface pressure as in the absence of cholesterol, and a similar value (≈24 mN/m) for the limiting pressure π_c is observed. Notably, for eggPC/β-cholesterol film, the value of π_0 at which the mechanism of interaction of CsA with lipid monolayer alters (change in slope for the $\Delta\pi$ versus π_0 graph) increases to approximately 22 mN/m. The extrapolation of the penetration of CsA into the more densely packed films yields π_c of ≈31 mN/m for eggPC/β-cholesterol monolayers, whereas $\pi_{c\ of}$ ≈35 mN/m was measured for the eggPC film. To this end, the above effects of CsA were not stereospecific, and similar data were measured for α-cholesterol containing monolayers (data not shown).

Differential Scanning Calorimetry. Previous DSC studies have revealed CsA to decrease the enthalpy (ΔH) of the main phase transition of DPPC, and a location of the drug in the hydrophobic membrane interior was suggested (O′Leary et al., 1986; Wiedmann et al., 1990). A similar effect is observed for cholesterol (Demel and de Kruyff, 1976) and is shown at X_{chol} = 0.1 for a binary mixture with DMPC (Fig. 3). CsA further broadens the main transition peak of DMPC/β-cholesterol (10:1, molar ratio) MLVs (Fig. 3), suggesting possible intercalation of CsA within the hydrocarbon interior of the membrane. The values ΔH for for DMPC/β-cholesterol (10:1, mol/mol) with progressively increasing contents of CsA are depicted in Fig. 4A. The main transition enthalpy (denoted as ΔH_0) for the DMPC/β-cholesterol MLVs is approximately 11 kJ/mol. At drug-to-lipid ratios ranging from 1:100 to 2:100, CsA increases ΔH by approximately 2 kJ/mol. At drug-to-lipid stoichiometry of 3:100, a sharp dip in ΔH is observed, with ΔH decreasing to 9.1 kJ/mol, yet on further increasing drug-to-lipid stoichiometry to 7:200, ΔH increases. The value for ΔH remains above ΔH_0 at drug-to-lipid ratios of 4:100 and 9:200, whereas a sharp minimum in ΔH is observed at drug-to-lipid ratios of 5:100 and 11:200, with the ΔH being approximately 50% of ΔH_0. At drug-to-lipid molar

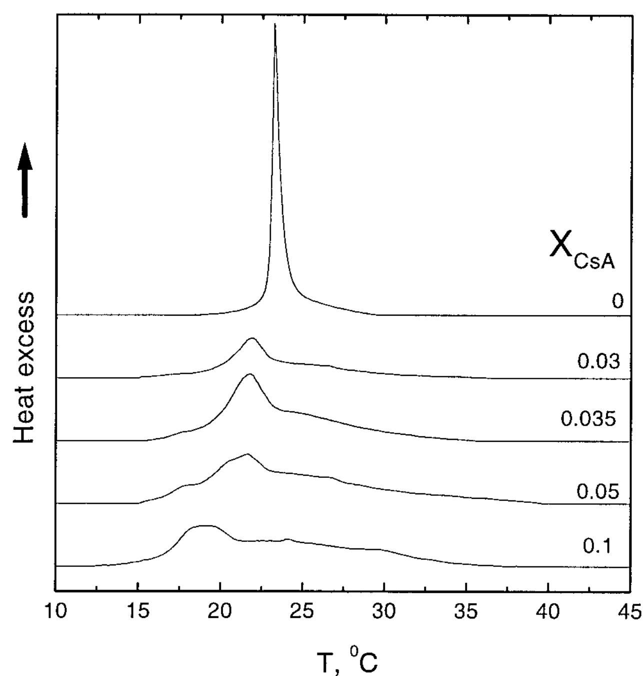
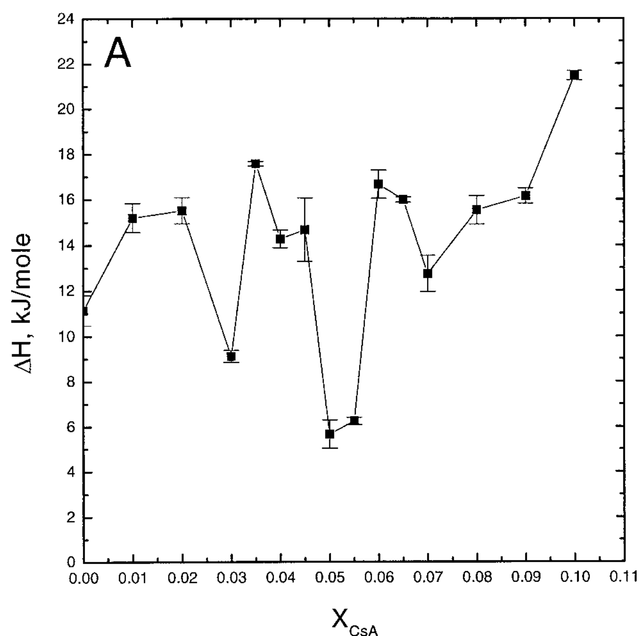


Fig. 3. Broadening of the main phase transition peak of DMPC: β-cholesterol (10:1, mol/mol) MLVs on increasing X_{CsA}, measured by DSC. Total lipid concentration was 0.77 mM in 5 mM HEPES and 0.1 mM EDTA, pH 7.4. The heating rate was 0.5°C/min.



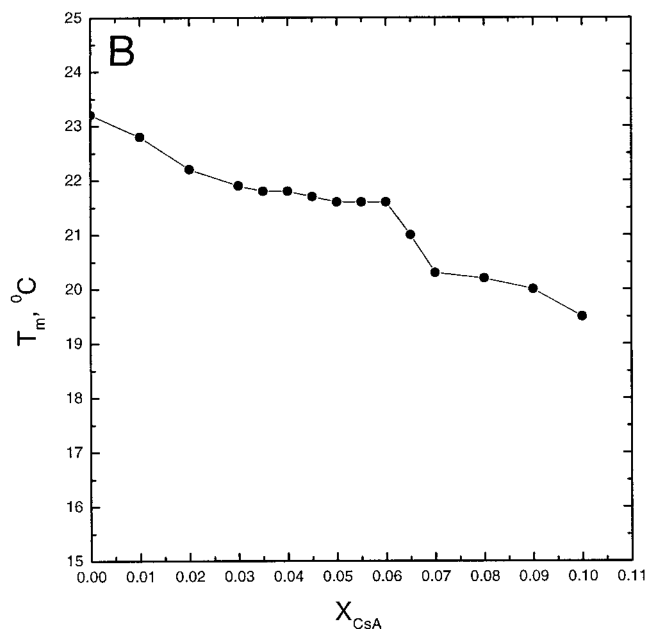


Fig. 4. A, Changes in the main ΔH of DMPC/β-cholesterol (10:1, mol/mol) MLVs due to increasing concentrations of CsA. Total lipid concentration was 0.77 mM in 5 mM HEPES and 0.1 mM EDTA, pH 7.4. All samples had the same thermal history. Scanning rate was 0.5°C/min. Each data point represents the mean of triplicate analyses, with the error bars indicating ±S.D. B, Changes in the peak of the maximum heat capacity (T_m) of DMPC/β-cholesterol (10:1, mol/mol) MLVs due to increasing concentrations of CsA.

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ratios of 6:100 and 13:200, ΔH again exceeds ΔH_0, where after a 7:100 drug-to-lipid ratio, a smaller dip in ΔH is observed. As the content of CsA is further increased up to drug-to-lipid stoichiometry of 10:100, the value for ΔH increases, becoming nearly twice the value of ΔH_0. Less dramatic changes are evident in T_m (Fig. 4), yet on exceeding X_{CsA} = 0.06, there is a decrement in T_m by approximately 0.6 degree.

Fluorescence Microscopy of the Monolayers. Morphology of the two-dimensional domains of lipid monolayers is sensitive to their chemical composition (e.g., Weis and McConnell, 1985; Weis, 1991). To obtain further insight into the effects of CsA on lipid alloys, we studied the lateral distribution of the fluorescent lipid analog NBD-PC (X = 0.02) in DPPC monolayers as a function of π. Representative images of a DPPC monolayer at surface pressures of 13.1 and 18.7 mN/m are shown in Fig. 5, A and C. These pressures correspond to the transition region, evident as the coexistence of the fluid and solid regions. As the fluorescent probe, NBD-PC readily partitions into the former domains; these appear as light and dark areas, respectively (Weis and McConnell, 1985). In the absence as well as the presence of CsA, dark, crystalline domains containing very low amounts of probe first appeared on compression to surface pressures between 9 and 10 mN/m (Fig. 5B). The presence of CsA (X = 0.05) diminishes domain size, whereas the length of the fluid/gel (liquid-expanded/liquid-condensed) domain boundary increases. At higher pressures, the effect of CsA on the domain boundaries becomes more pronounced (Fig. 5B). For example, for DPPC/NBD-PC film at 18.7 mN/m, the domain boundaries are diffuse, with a gradient of decreasing NBD-PC fluorescence on going from crystalline to fluid region (Fig. 6). This is contrasted by the sharp boundaries observed in the presence of CsA (Fig. 6). The shape of the domains observed with CsA somewhat resembles those reported for low mole fractions (X = 0.02) of cholesterol (Weis and McConnell, 1985).

The above results suggest that CsA might have a preference for fluid/gel domain boundaries, so as to stabilize these (Weis and McConnell, 1985). Accordingly, we investigated the effects of CsA on DPPC films containing cholesterol (X = 0.10). Representative images are compiled in Fig. 7. In the absence of CsA and at surface pressures in the range of 13.3 to 30.5 mN/m, the distribution of NBD-PC fluorescence produces very complex patterns with varying fluorescence intensities (A– D). The effect of CsA (X = 0.05) on the observed patterns is striking (E–H). In brief, over the same range of surface pressure, only circular dark domains with well-defined boundaries are seen.

Discussion

Conventionally, drugs are a priori assumed to exert their action in cells by more or less specific binding to sites in proteins. Interestingly, a novel concept has emerged from studies on the membrane binding of cytotoxic peptides such as magainins, cecropins, and defensins (Bechinger, 1997). No receptors have been demonstrated for these compounds, and they are presently believed to exert their activity by interacting with the lipid bilayer. Yet, the molecular mechanism or mechanisms of their action and the property of the bilayer being modified remain to be established. In this regard, the rich scale of different phases (i.e., membranes with distinct physicochemical properties) exhibited by different lipids is of interest (Kinnunen and Laggner, 1991). There is experimental evidence indicating a correlation between the physical state (i.e., the phase state of cellular membranes), determined by their lipid composition, and the physiological state

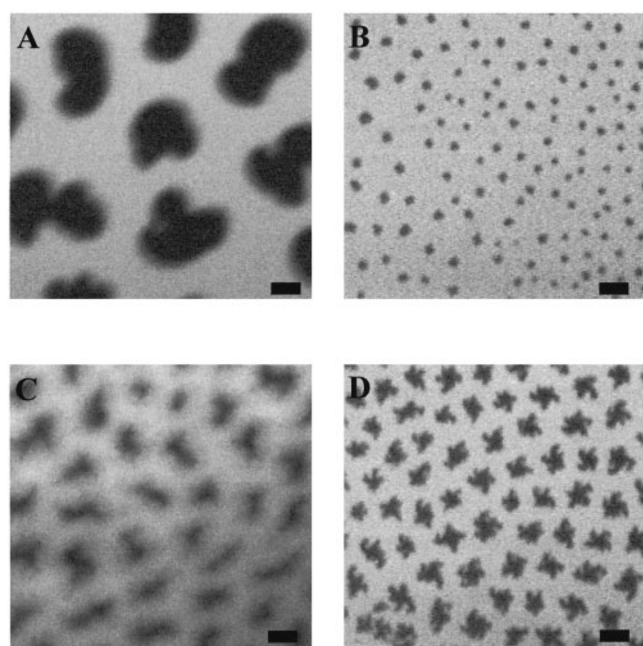

Fig. 5. Fluorescence microscopy images of DPPC/NBD-PC (98:2, mol/mol) monolayers in the absence (A and C) and presence of CsA (X = 0.05; B and D). The subphase is 5 mM HEPES and 0.1 mM EDTA, pH 7.4, at ambient temperature (~+24°C). The compression rate was 1 Å²/acyl chain/min. Values of surface pressure were (A) 13.1 mN/m, (B) 14.1 mN/m, (C) 18.7 mN/m, and (D) 17.8 mN/m. Scale bar is 20 μm. See text for more details.

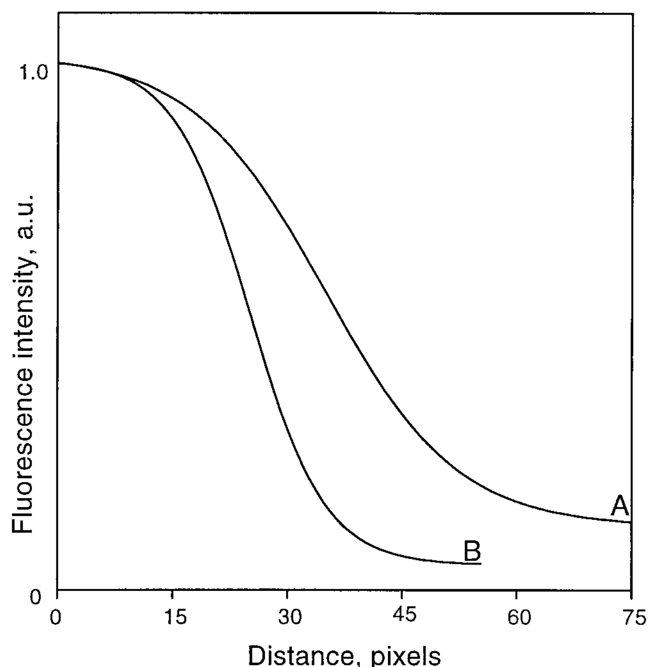

Fig. 6. Effect of CsA on the lateral distribution profile of NBD fluorescence intensity within the domain boundaries for DPPC/NBD-PC (98:2, mol/mol) films. The data were taken from C (no CsA, graph A) and D (X_{CsA} = 0.05, graph B) in Fig. 5 and were fitted by using sigmoidal function. For both graphs, the data were normalized to one measured for the fluid domain.

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of the cell (Kinnunen, 1991, 1996). There is no reason to believe that the modulation of specific properties of the lipid bilayer would be limited to the above cytotoxic peptides; it could contribute to both the therapeutic mechanism and side effects of membrane-partitioning compounds in general. To this end, a large variety of structurally dissimilar drugs are hydrophobic or amphiphilic and readily partition into lipid membranes; good examples are tacrine (Lehtonen et al., 1996), doxorubicin (Mustonen et al., 1993; Söderlund et al., 1999), and CsA (O′Leary et al., 1986; Wiedmann et al., 1990). Drugs may also modulate peripheral lipid-protein interactions, as shown for chlorpromazine, doxorubicin, lidocaine, and gentamycin (Ito et al., 1983; Mustonen and Kinnunen, 1991; Jutila et al., 1998).

The increase in acyl chain order in DPPC liposomes by CsA is similar to that caused by cholesterol (Wiedmann et al., 1990). Increasing the contents of cholesterol progressively decreases ΔH of the main transition in a manner depending on the acyl chain composition of the matrix lipid (McMullen et al., 1993). Comparison of these data on the effects of cholesterol and CsA suggests the localization of these compounds in membranes to be similar. For cholesterol, the long axis of its sterol ring structure parallels the acyl chains of the membrane lipids, with the hydroxyl group residing vicinal to the phospholipid ester carbonyl groups. The side chain is buried in the membrane interior. CsA occupies a larger area in the interior of the membrane compared with the membrane surface (Wiedmann et al., 1990). Also, the present DSC data are consistent with the intercalation of CsA into the membrane interior (O′Leary et al., 1986; Wiedmann et al., 1990).

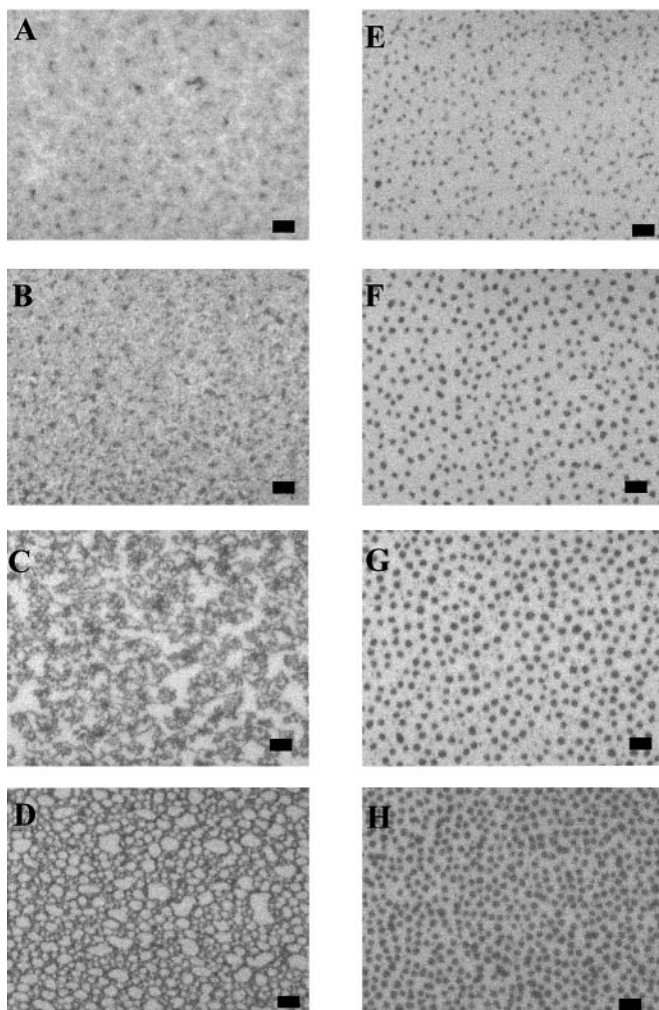

Fig. 7. Changes in the NBD-PC (X = 0.02) distribution in DPPC/β-cholesterol (88:10, mol/mol) monolayers caused by CsA, observed by fluorescence microscopy. The lipid alloy organization is shown in the absence (A–D) and the presence of CsA (X = 0.05, E–H). Surfaces pressures were (A) 13.3 mN/m, (B) 18.8 mN/m, (C) 19.6 mN/m, (D) 30.5 mN/m, (E) 13.8 mN/m, (F) 17.7 mN/m, (G) 22.1 mN/m, and (H) 31.4 mN/m. Subphase was 5 mM HEPES and 0.1 mM EDTA, pH 7.4. Scale bar is 20 μm. See text for further details.

The thermal phase behavior of DMPC/β-cholesterol (10:1, mol/mol) liposomes as a function of X_{CsA} is complex (Fig. 4). A likely explanation to these data could be provided by the same principles as forwarded for tacrine-induced changes in the thermal behavior of dimyristoylphosphatidic acid (Lehtonen et al., 1996). More specifically, the latter results were interpreted in the terms of formation of regular superstructured regions in the bilayer at well-defined drug-to-phospholipid ratios. In principle, all systems organize so as to minimize their free energy. In a bilayer alloy, this may require its components to respond to changes in composition by changes in organization. This is best implied by the alterations observed in the main transition enthalpy at CsA-to-cholesterol ratios of 3:10 and 1:2 (X_{CsA} = 0.03 and 0.05, respectively). The formation of superlattices in liposomes containing cholesterol has been proposed (Liu et al., 1997). CsA could exert effects similar although not identical to those of cholesterol (Wiedmann et al., 1990).

The biphasic, packing density-dependent interaction of CsA with the phospholipid monolayer is peculiar and reveals cholesterol to decrease the penetration of CsA into the lipid monolayer in a surface pressure-dependent manner (i.e., at pressures exceeding 22 mN/m) (Fig. 2). Because the inclusion of cholesterol leads to a increased lateral packing density of phosphatidylcholine monolayers (Gershfeld and Pagano, 1972), it also decreases the free volume within the hydrophobic part of the monolayer. Obviously, this would impede the penetration of CsA into the lipid, yet more specific lipid-drug interactions also could be involved and could reflect a pressure-induced change in the conformation and/or orientation of CsA. In this case, the conformation and/or orientation of CsA in the membrane would also be sensitive to cholesterol.

Fluorescence microscopy of lipid monolayers has been used to study the lateral organization of lipids (Nag et al., 1991), changes in lipid domains induced by phospholipase A_2 (Grainger et al., 1989), monolayer phase transitions (Weis, 1991), domain formation induced by Ca^{++} (Eklund et al., 1988), and domain formation induced by electric fields (Lee et al., 1994). Lipid-protein interactions have also been studied by fluorescence microscopy of monolayers (Subirade et al., 1995). The determinants for domain morphologies has been a subject of intense research (Weis, 1991), and factors such as dipole fields and line tension have been shown to contribute. To our knowledge, this technique has not been used to investigate possible changes in domain morphology caused by drugs. The fluorescent probe NBD-PC used in this study preferentially partitions into the "fluid" (liquid-expanded) lipid (Weis and McConnell, 1985). Accordingly, fluorescence microscopy of phospholipid monolayers allows visualization of the coexistence of fluid and gel domains in the transition region (Weis, 1991). In the transition region, three phases

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with distinctly different fluorescence intensities are evident (Fig. 7). The dark and light areas represent gel-like and fluid domains, respectively, separated by a boundary region with a gray, intermediate, and diffuse gradient of fluorescence emission. Evidence for an "intermediate" lipid domain within the main transition has been recently forwarded for DMPC liposomes (Jutila and Kinnunen, 1997). More complex behavior is observed when β-cholesterol is present. DPPC/β-cholesterol/NBD-PC monolayers exhibit reticular domains at higher surface pressures. The effect of CsA on the lateral organization of this system is dramatic, and only gel- and fluid-like domains are evident. The change in the domain shape is also pronounced, from complex reticular networks to completely circular domains at all surface pressures. The effect of CsA on the lateral organization of pure DPPC monolayers suggests that it partitions into the boundaries between gel/liquid domains, with these boundaries becoming sharp in the presence of this drug.

The present results show that the interaction of CsA with membranes containing cholesterol are much more complex than those revealed in previous studies with neat PC bilayers (Wiedmann et al., 1990). In brief, we demonstrated that CsA not only changes the thermal phase behavior of the membrane but also alters dramatically the lateral organization in monolayers on a micrometer scale. Although direct comparison of the different membrane models, liposomes and monolayers, is ambiguous, we can conclude that both systems demonstrate that the interaction of CsA with membrane lipids depend on the presence of cholesterol. There is a large body of evidence showing that cholesterol affects a number of processes of diverse nature in different cells. Moreover, organization of cholesterol in membranes can be anticipated to be critical to its functions (see Schroeder et al., 1995, and references therein). Definitive conclusions regarding the pharmacological significance of our findings would clearly be premature at this stage, yet in conjunction with the importance of coupling between organization and function in biomembranes, the present results do indicate that efforts along these lines may provide novel insights to the understanding of the molecular mechanisms of action of membrane-associating drugs.

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References

Bechinger B (1997) Structure and functions of channel-forming peptides: Magainins, cecropins, mellitin and alamethicin. *J Membr Biol* **156**:197–211.

Bernardi P (1996) The permeability transition pore: Control points of a cyclosporin A-sensitive mitochondrial channel involved in cell death. *Biochim Biophys Acta* **1275:**5–9.

Cohen DJ, Loertscher R, Rubin MF, Tilney NL, Carpenter CB and Strom TB (1984) Cyclosporine: A new immunosuppressive agent for organ transplantation. *Ann Intern Med* **101:**667–682.

Demel RA and de Kruyff B (1976) The function of sterols in membranes. *Biochim Biophys Acta* **457:**109–132.

de-Paula E and Schreier S (1996) Molecular and physicochemical aspects of local anesthetic-membrane interaction. *Brazilian J Med Biol Res* **29**:877–894.

Eklund KK, Vuorinen J, Mikkola J, Virtanen JA and Kinnunen PKJ (1988) Ca^{2+}-induced lateral phase separation in phosphatidic acid/phosphatidylcholine monolayers as revealed by fluorescence microscopy. *Biochemistry* **27:**3433–3437.

Epand RM, Epand RF and McKenzie RC (1987) Effects of viral chemotherapeutic agents on membrane properties: Studies of cyclosporin A, benzyloxycarbonyl-D-Phe-L-Phe-Gly and amantadine. *J Biol Chem* **262:**1526–1529.

Gershfeld NL and Pagano RE (1972) Physical chemistry of lipid films at the air-water interface. III. The condensing effect of cholesterol: A critical examination of mixed-film studies. *J Physiol Chem* **76:**1244–1249.

Goormaghtigh E, Brasseur R and Ruysschaert JM (1982) Adriamycin inactivates cytochrome *c* oxidase by exclusion of the enzyme from its cardiolipin essential environment. *Biochim Biophys Res Commun* **104:**314–320.

Grainger DW, Reichert A, Ringsdorf H and Salesse C (1989) An enzyme caught in action: Direct imaging of hydrolytic function and domain formation of phospholipase A_2 in phosphatidylcholine monolayers. *FEBS Lett* **252:**73–82.

Hemar A and Dautry-Varsat A (1990) Cyclosporin A inhibits the interleukin 2 receptor alpha chain gene transcription but not its cell surface expression: The alpha chain stability can explain this discrepancy. *Eur J Immunol* **20:**2629–2635.

Ipsen JH, Karlström G, Mouritsen OG, Wennerström H and Zuckermann MJ (1987) Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim Biophys Acta* **905:**162–172.

Ito S, Werth DK, Richert ND and Pastan I (1983) Vinculin phosphorylation by the *src* kinase: Intercation of vinculin with phospholipid vesicles. *J Biol Chem* **258:**14626–14631.

Jutila A and Kinnunen PKJ (1997) Novel features of the main transition of dimyristoylphosphocholine bilayers revealed by fluorescence spectroscopy. *J Phys Chem* **101:**7635–7640.

Jutila A, Rytömaa M and Kinnunen PKJ (1998) Detachment of cytochrome *c* by cationic drugs from membranes containing acidic phospholipids: Comparison of lidocaine, propranolol, and gentamycin. *Mol Pharmacol*, **54:**722–732.

Kitagaki K, Nagai H, Hayashi S and Totsuka T (1997) Facilitation of apoptosis by cyclosporins A and H, but not FK506 in mouse bronchial eosinophils. *Eur J Pharmacol* **337:**283–289.

Kinnunen PKJ (1991) On the principles of functional ordering in biological membranes. *Chem Phys Lipids* **57:**375–399.

Kinnunen PKJ (1996) On the molecular-level mechanisms of peripheral protein-membrane interactions induced by lipids forming inverted non-lamellar phases. *Chem Phys Lipids* **81:**151–166.

Kinnunen PKJ and Laggner P, eds (1991) Phospholipid phase transitions. *Chem Phys Lipids* **57:**109–399.

Kopp JB and Klotman PE (1990) Cellular and molecular mechanisms of cyclosporin nephrotoxicity. *J Am Soc Nephr* **1:**162–179.

Lance MR, Washington C and Davis SS (1996) Evidence for the formation of amphotericin B-phospholipid complexes in Langmuir monolayers. *Pharmaceut Res* **13:**1008–1014.

Lee KYC, Klinger JF and McConnell HM (1994) Electric field-induced concentration gradients in lipid monolayers. *Science (Wash DC)* **263:**655–658.

Lehtonen JYA, Rytömaa M and Kinnunen PKJ (1996) Characteristics of the binding of tacrine to acidic phospholipids. *Biophys J* **70:**2185–2194.

Liu F, Sugar IP and Chong PLG (1997) Cholesterol and ergosterol superlattices in three-component liquid crystalline lipid bilayers as revealed by dehydroergosterol fluorescence. *Biophys J* **72:**2243–2254.

McMullen TPW, Lewis RNAH and McElhaney RN (1993) Differential scanning calorimetric studies of the effect of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated phosphatidylcholines. *Biochemistry* **32:**516–522.

Mouritsen OG and Kinnunen PKJ (1996) Role of lipid organization and dynamics for membrane functionality, in *Biological Membranes* (Merz K Jr and Roux B eds) pp 463–502, Birkhäuser, Boston.

Mustonen P and Kinnunen PKJ (1991) Activation of phospholipase A_2 by adriamycin in vitro: Role of drug/lipid interactions. *J Biol Chem* **266:**6302–6307.

Mustonen P, Lehtonen JYA and Kinnunen PKJ (1993) Effects of sphingosine on peripheral membrane interactions: Comparison of adriamycin, cytochrome *c*, and phospholipase A_2. *Biochemistry* **32:**5373–5380.

Mustonen P, Virtanen JA, Somerharju PJ and Kinnunen PKJ (1987) Binding of cytochrome *c* to liposomes as revealed by the quenching of fluorescence from pyrene-labelled phospholipids. *Biochemistry* **26:**2991–2997.

Nag K, Boland C, Rich N and Keough KMW (1991) Epifluorescence microscopic observation of monolayers of dipalmitoylphosphatidylcholine: Dependence of domain size on compression rates. *Biochim Biophys Acta* **1068:**157–160.

O'Donohue MF, Burgess AW, Walkinshaw MD and Treutlein HR (1995) Modeling conformational changes in cyclosporin A. *Prot Sci* **4:**2191–2202.

O'Leary TJ, Ross PD, Lieber MR and Levin IW (1986) Effects of cyclosporine A on biomembranes: Vibrational spectroscopic, calorimetric and hemolysis studies. *Biophys J* **49:**795–801.

Rytömaa M and Kinnunen PKJ (1996) Dissociation of cytochrome *c* from liposomes by histone H1: Comparison with basic peptides. *Biochemistry* **35:**4529–4539.

Schroeder F, Woodford JK, Kavecansky J, Wood WG and Joiner C (1995) Cholesterol domains in biological membranes. *Mol Membr Biol* **12:**113–119.

Söderlund T, Jutila A and Kinnunen PKJ (1999) Binding of adriamycin to liposomes as a probe for membrane lateral organization. *Biophys J* In Press.

Subirade M, Salesse C, Marion D and Pezolet M (1995) Interaction of a nonspecific wheat lipid transfer protein with phospholipid monolayers imaged by fluorescence microscopy and studied by infrared spectroscopy. *Biophys J* **69:**974–988.

Weis RM (1991) Fluorescence microscopy of phospholipid monolayer phase transitions. *Chem Phys Lipids* **57:**227–239.

Weis RM and McConnell HM (1985) Cholesterol stabilizes the crystal-liquid interface in phospholipid monolayers. *J Physiol Chem* **89:**4453–4459.

Wiedmann TS, Trouard T, Shekar SC, Polikandritou M and Rahman YE (1990) Interaction of cyclosporin A with dipalmitoylphosphatidylcholine. *Biochim Biophys Acta* **1023:**12–18.

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