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ARTICLE *in* BIOCHEMISTRY · JANUARY 2004

Impact Factor: 3.02 · DOI: 10.1021/bi035759c · Source: PubMed

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Steroid Structural Requirements for Stabilizing or Disrupting Lipid Domains†

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Received September 29, 2003

ABSTRACT: In artificial membrane bilayers, saturated long acyl chain-containing phospholipids and cholesterol (Chol) interact to form more ordered domains than those in phospholipids with unsaturated or short fatty acyl chains. We have extended the fluorescence techniques of London et al. [Xu, X., and London, E. (2000) *Biochemistry 39*, 843−849; Xu, X., Bittman, R., Duportail, G., Heissler, D., Vilchezes, C., and London, E. (2001) *J. Biol. Chem. 276*, 33540−33546] to study the propensity of several steroids to form or disrupt such ordered lipid domains. Temperature-dependent fluorescence quenching and steady-state polarization of the extrinsic fluorescent probe diphenylhexatriene (DPH) in model membranes composed of dipalmitoylphosphatidylcholine (or sphingomyelin), a nitroxide spin-labeled phosphatidylcholine (12-SLPC), and a given steroid were combined to study the influence of the latter on (a) ordered lipid domain formation, (b) stabilization, and (c) the extension of the ordered lipid assemblies. The results of the two totally independent methods, fluorescence quenching by 12-SLPC and fluorescence polarization of DPH, show that all steroids examined, except for Chol and 25-hydroycholesterol, behave as lipid domain-disrupting compounds. Additionally, we found a positive correlation between the hydrophobicity of steroids and their ordered lipid domain-promoting activity. Comparison of the chemical structures disclosed some distinctive traits of ordered lipid domain-promoting steroids: (i) the presence of an isooctyl side chain bond at C17; (ii) the absence of carbons attached to C23 (i.e., C24−C27) in any of the other (domain-disrupting) steroids; (iii) the presence of a small polar group at position C3; and (iv) the absence of polar groups in the fused rings, with the exception of substitutions at position C3 in the A ring.

Subcellular fractions insoluble in nonionic detergents at 4 °C and enriched in cholesterol (Chol),[1] sphingomyelin (Sph), and complex glycosphingolipids (GSL) and exhibiting a light buoyant density in sucrose gradients were operationally defined as lipid "rafts" (*3*, *4*) and are postulated to be involved in biological functions such as sorting of membrane components, membrane signaling, viral budding, amyloid formation, and biosynthetic and endocytic traffic (*5*−*12*). The presence and the possible function of such domains in biological membranes in general are therefore of great interest. In addition to their lipid content, rafts display varying protein compositions, being particularly enriched in glycosylphosphatidylinositol- (GPI-) linked proteins anchored by saturated acyl chains but are poor in transmembrane proteins, which seldom occur in lipid rafts (*13*).

Lipid bilayers may be found in several physical states depending on a number of physicochemical factors. The solid-gel phase (L_β) is characterized by tight phospholipid acyl chain packing and by restricted lateral motion, whereas the fluid liquid-crystalline (L_α, L_R) phase, typically associated with lipid domains enriched in naturally occurring phospholipids with predominantly unsaturated acyl chains (*14*−*16*), displays a less ordered lipid packing and exhibits faster lateral diffusion. A third phase, the so-called liquid-ordered phase (L_o, or γ state in early studies), is found in mixtures of highly saturated phospholipids and Chol and in Sph−Chol mixtures in particular (*15*−*20*). This phase shares properties with the L_α and L_β phases: lipids are well packed in an ordered state similar to that of the L_β, although they display lateral diffusion almost as fast as in the L_α phase (see review in ref *21*). The tightly packed, relatively ordered state of lipids in the L_o phase has been associated with detergent insolubility, relevant to the operational definition of raft, although such equivalence is open to discussion (see, e.g., refs *22* and *23*). When the physicochemical conditions change, the L_o phase may undergo a transition toward the disordered phase (L_d) and vice versa.

In artificial membranes at low temperatures, lipids of different transition temperatures (T_m) may segregate, giving rise to the appearance of two coexisting phases enriched in one of the lipids (*21*). The physical state of the bilayer and the structure of segregated domains are influenced by at least two factors: composition (saturated lipids favor L_o phases and domain formation, whereas unsaturated lipids favor the opposite) and temperature (the lower the temperature, the higher the probability or the extent of domain formation)

† This work was supported in part by grants from the Universidad Nacional del Sur, the Agencia Nacional de Promoción Científica (FONCYT), Argentina, and FIRCA 1-RO3-TW01225-01 (NIH) to M. White and F.J.B.

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[1] Abbreviations: Chol, cholesterol; DPH, diphenylhexatriene; GPI, glycosylphosphatidylinositol; GSL, glycosphingolipids; DFSC, domain formation/stabilization coefficient; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; 25-OH-Chol, 25-hydroxycholesterol; MLV, multilamellar vesicles; *P*, fluorescence polarization; *Q*, fluorescence quenching; Sph, sphingomyelin; 12-SLPC, 12-nitroxide-labeled 1-palmitoyl-2-stearoyl-(12-doxyl)-*sn*-glycero-3-phosphocholine; T_m, phase transition temperature.

10.1021/bi035759c CCC: $25.00
Published on Web 11/11/2003

(*1*, *2*, *16*). Some compounds can either promote or inhibit this segregation of components by influencing the packing of the saturated lipid. Chol, at certain concentrations, has been reported to promote separation of lipid mixtures into coexisting L_d and (detergent-insoluble) L_o domains, although little is known on the effects of other biologically relevant sterols such as steroid hormones (*24*). In living cells, Chol is purported to promote lipid domain formation and influence both the association with lipid domains and the function of proteins that are found in sucrose gradients together with the so-called detergent-resistant membranes (*25*, *26*).

The aim of the present work is to investigate the effect of several natural and synthetic steroids, some of them with important physiological and clinical effects, on ordered lipid domain formation/stabilization in simple model systems. For this purpose, we resort to the fluorescence assay of London and co-workers (*1*, *2*) to evaluate the ability of different steroids to either promote or disrupt ordered lipid domain formation. In this method, the degree of fluorescence quenching of DPH by a nitroxide spin-labeled phosphatidylcholine in saturated lipid-containing liposomes is compared to the degree of quenching in unsaturated lipid-containing liposomes. Here we modify and extend these fluorescence techniques, and using two totally independent methods, fluorescence quenching by a spin-labeled phospholipid and fluorescence polarization of the extrinsic fluorescent probe DPH, we extract information on structural features influencing ordered lipid domain formation, stabilization, or disruption. Furthermore, the lipid domain-promoting or -disrupting ability of various steroids is correlated with their hydrophobicity and molecular structure.

EXPERIMENTAL PROCEDURES

Materials. Dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), diphenylhexatriene (DPH), and all steroids were purchased from Sigma Chemical Co. (St. Louis, MO). Nitroxide-labeled 1-palmitoyl-2-stearoyl-(12-doxyl)-*sn*-glycero-3-phosphocholine (12-SLPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Stock solutions were prepared in chloroform/methanol (2:1 v/v) at a final concentration of 1 mg/mL in the case of phospholipids and quencher, 0.5 mg/mL in the case of steroids, and 0.01 mg/mL for DPH. The fluorescent probe was first dissolved in tetrahydrofuran and subsequently diluted in chloroform/methanol. All solutions were stored at −20 °C before use.

Liposome Preparation. Multilamellar vesicles (MLV) were prepared by mixing the appropriate amount of stock solution of each component (Table 1) in round-bottomed tubes and drying under nitrogen at 25−30 °C while rotating the tube. The samples were then further dried under nitrogen for 1 h to eliminate the remaining solvent. Buffer (20 mM Hepes, 150 mM NaCl, 0.25 mM $MgCl_2$, pH 7.4) was added to the thin lipid film, and the mixture was heated to 48−50 °C (i.e., at a temperature higher than the highest T_m of the constitutive lipids). At this stage, the total lipid concentration of the suspension (including steroids) was 0.5 mg/mL. The samples were then vigorously mixed in a Vortex mixer in two steps of 1 min each, also at 48−50 °C, and incubated for 30 min in a low-power bath sonicator in order to obtain a more uniform size distribution of the resulting MLVs.

Table 1: Relative Lipid and Fluorescent Probe Concentrations in the Liposomes Used in Fluorescence Experiments[a]

	mol %				
	background fluorescence sample	DPPC samples		DOPC samples	
		without quencher	with quencher	without quencher	with quencher
DPH		1	1	1	1
DPPC	48	48	48		
DOPC	32	32		80	48
12-SLPC			32		32
steroid	20	20	20	20	20

[a] The relative concentrations listed in this table apply to all experiments, except for those of Figure 2, which contained 30 mol % of Chol instead of 20 mol %. Control samples differed only in their lack of steroid. Total lipid concentration (including steroids) was 50 μM, and the quencher:lipid molar ratio was kept at 40:60 in all experiments. The composition of samples in the experiments described in Figure 5 was identical to those listed in the table, except that DPPC was replaced by Sph.

Samples were diluted with buffer to a final concentration of 50 μM and kept at 4 °C in the dark for a period no longer than 20 h. For fluorescence measurements, two aliquots were separated for the quenching and polarization assays. Background fluorescence was measured in liposomes containing the same steroid and lipids but having no quencher and no DPH. In all cases, the background fluorescence intensity amounted to less than 6% of that of its fluorescence probe-containing counterpart (Table 1).

Fluorescence Measurements. Fluorescence emission spectra and polarization were recorded using an SLM 4800 ISC spectrofluorometer (SLM Instruments, Urbana, IL). Optimal excitation/emission wavelengths for DPH were 358 and 424 nm, respectively. Measurements were carried out in 10 × 10 mm quartz cuvettes at increasing temperatures (10−55 °C). A 418 nm cutoff filter (KV418; Schott, Mainz, Germany) was used for the polarization measurements. The heating rate was 0.5 °C/min. Fluorescence measurements were carried out in triplicate of independent experiments 5−10 min after reaching the desired working temperature, which was regulated by a thermostated circulating water bath (Haake, Darmstadt, Germany) and monitored inside the cuvette with a thermocouple. Background fluorescence was subtracted from each sample before the fluorescence quenching values were calculated.

RESULTS AND DISCUSSION

Rationale. The strategy behind the experimental approach used here to assess lipid domain formation or disruption is based on the comparison between the degree of quenching induced by a nitroxide spin-labeled phospholipid, 12-SLPC, on the extrinsic fluorescent probe DPH reconstituted in DPPC- and DOPC-containing liposomes, respectively (*1*, *2*). When membranes are made up of an unsaturated lipid (e.g., DOPC) and a fluorescent quenching lipid (e.g., 12-SLPC) with a phase behavior similar to that of an unsaturated lipid (the T_m of both lipids is below 0 °C), all components mix and distribute randomly in the bilayer at temperatures above their T_m (left panel in Figure 1). Thus, no segregation occurs between lipid and quencher (12-SLPC), and at the concentration of species used, the majority of the DPH molecules are expected to have a quencher molecule in their vicinity.

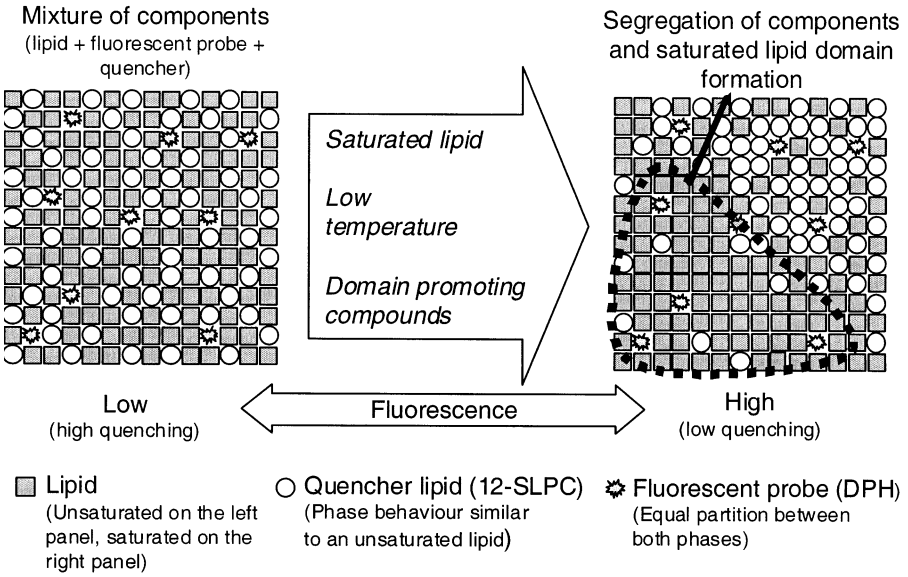

FIGURE 1: Scheme illustrating the principle of the quenching method used for domain detection in model membranes. Left: membrane components are distributed randomly (because of unsaturated lipid composition, high temperature, inappropriate lipid/quencher relationship, and/or the presence of disrupting lipids). Right: segregation of lipids (produced by the change of one or more of the above-mentioned factors) leads to an increase in the fluorescence signal because of the diminished quenching.

Consequently, the fluorescence of the system as a whole is highly quenched.

When the unsaturated lipid is replaced by a saturated lipid (e.g., DPPC) and all other components remain the same (right panel in Figure 1), DPPC molecules segregate from the quencher molecules at temperatures below their T_m. Since DPH displays no preference for either phase, it distributes itself almost equally between the two (*27*); the population of DPH molecules remaining in the DPPC-enriched domain will exhibit a lower probability of interacting with quencher molecules, and consequently, the degree of quenching is low and the measured fluorescence intensity is high.

A necessary requisite for DPH to operate as a faithful probe in this system is that its quantum yield does not depend on the phase state of the surroundings in any lipid phase. We experimentally determined that the fluorescence intensity of DPH in DPPC/cholesterol liposomes did not significantly change in the 8−51 °C temperature range, suggesting that phase transition does not affect the quantum yield of DPH (data not shown), in agreement with data of Lenz et al. (*27*) on DPPC/DMPC liposomes.

Comparative Phase Behavior of DOPC/12-SLPC/Chol and DPPC/ 12-SLPC/ Chol. For segregation to occur, there is an optimal relationship between the concentrations of lipid species in a mixture. If the molar ratio of any of the components is low enough with respect to the other, the lipid will remain dissolved in the other lipid and no segregation will occur. Thus, outside a certain lipid/quencher molar ratio, both lipids remain soluble in each other. The quenching of DPH in a series of DOPC/12-SLPC/Chol and DPPC/12-SLPC/Chol liposomes containing different lipid:quencher mole ratios was measured at various temperatures in order to determine the appropriate molar ratio at which the difference between the quenching in DPPC- and DOPC-containing liposomes was maximal, and a satisfactory fluorescence signal in the quenched samples was achieved. In preliminary experiments the Chol concentration was kept at 30 mol % with respect to [quencher + lipid]. Experimental

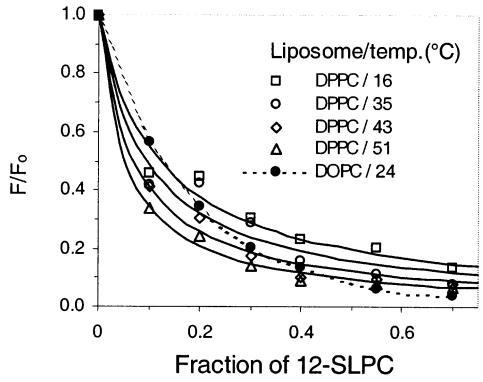

FIGURE 2: Comparison of quenching curves for mixtures of DOPC/12-SLPC/Chol and DPPC/12-SLPC/Chol plotted against quencher/lipid molar fraction at different temperatures. Only the curve corresponding to 24 °C is shown for the DOPC-containing liposomes; all curves were superimposed with the former up to 52 °C. The total lipid concentration (including steroids) was 50 μM throughout this work. As an exception, in this series of experiments the Chol concentration was 30 mol % instead of the 20 mol % used elsewhere. The quencher:lipid molar ratio was 40:60. Experimental data were fitted with the Stern−Volmer equation.

quenching data were fitted following the Stern−Volmer equation: $F/F_0 = 1/(1 + K_D[Q])$, where K_D is the quenching constant and [Q] is the quencher concentration (Figure 2).

In the case of DOPC-containing liposomes, increasing the temperature from 24 to 52 °C did not give rise to any changes, and all curves were almost superimposed (for the sake of clarity, Figure 2 shows only the colder temperature for the DOPC-containing liposomes), indicating that the bilayer remained in the L_d phase at all temperatures. This is not unexpected in view of the transition temperature of DOPC ($T_m \cong -22$ °C). No domain segregation occurred between DOPC and 12-SLPC, and the level of quenching remained almost constant at all temperatures because DPH could not "hide" in any quencher-depleted domain.

The analysis of quenching curves of DPPC-containing liposomes showed a marked temperature-dependent quenching, i.e., lower F/F_0 values at high temperatures. At low

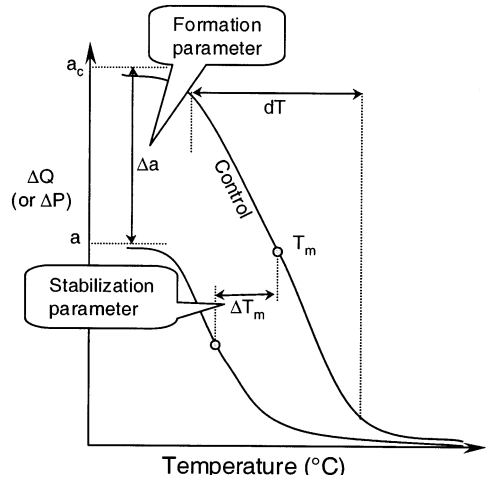

FIGURE 3: Parameters used in the calculation of DFSC (eq 3) in the proposed model (eq 2) (see text for details) to assess the ability of steroids to promote or disrupt lipid domain formation/stabilization. T_m is the transition temperature of the systems, dT is the difference between the temperature at which ΔQ (or ΔP) is 20% and 80%, respectively, of a, which is the highest asymptote. $(dT)^{-1}$ gives a measure of the slope near T_m. The formation [$\Delta a/a$] and stabilization [$\Delta T_m/T_m$] parameters are assumed to reflect the extent of the DPPC-rich domain and its thermal stability, respectively.

temperatures, DPPC is in the L_o phase and segregates from 12-SLPC (which is in the L_d phase within the temperature range studied), and fluorescence of DPH is then high due to the lower quencher concentration in the DPPC-enriched domain. At higher temperatures DPPC changes from the ordered to the disordered phase, lipid domains start to dissolve, and lipid molecules mix with the quencher molecules. This increases the degree of quenching because of the higher concentration of quencher molecules in the neighborhood of DPH molecules. Above a certain temperature, the whole system is in the L_d phase, and no further change in the quenching ratio is to be expected. At this stage, DPPC-containing liposomes are in a phase state like that of DOPC-containing liposomes (i.e., L_d). It should be noted that the temperature dependence of quenching in DPPC/12-SLPC mixtures is observed in the presence of domains but not in DOPC/12-SLPC mixtures that do not exhibit domain formation. This further indicates that the difference in quenching is not an artifact of 12-SLPC for quenching DPH in DPPC-containing mixtures but not in DOPC-containing mixtures. Since the difference between the fluorescence quenching of DPPC liposomes at low and high temperature (ΔQ) was evident within the entire quencher:lipid mole ratio studied (0.1−0.7), we used 40 mol % of 12-SLPC relative to DPPC for subsequent experiments designed to study the extent of the lipid domains.

Calculation and Modeling of Lipid Domain Formation/Stabilization from Quenching and Polarization Data. Plotting of DPH fluorescence quenching [$Q = F/F_0$] and fluorescence polarization [P] data in DPPC liposomes as a function of temperature generated sigmoid-shaped curves, which could be fitted by a Boltzman-type equation:

$$Q = \frac{(a_1 - a_2)}{1 + \exp\frac{(T - T_m)}{dT}} + a_2 \qquad (1)$$

where a_1 and a_2 are the highest and the lowest Q asymptotes, respectively, T is the temperature (°C), T_m is the transition temperature of the system (i.e., the temperature at which the domain extent is half of that at the lowest temperature), dT is the difference between the temperature at which Q constitutes 20% and 80%, respectively, of the difference between the highest and lowest asymptotes ($a_1 - a_2$), and its reciprocal (1/dT) gives a measure of the slope near T_m, the inflection point of the curve (see Figure 3). Nonlinear regression fitting (eq 1) of the experimental [Q] and [P] values yielded parameters for each sample replicate. The asymptotic [Q] (and [P]) experimental values at high temperatures were found to be different for the different steroids studied and different from zero in all cases. The curves were normalized to zero by subtracting the remaining constant quenching and polarization values at the highest temperatures (i.e., the a_2 parameter), also obtained by regression analysis (eq 1). The resulting ΔQ and ΔP values from each replicate were used to calculate the mean value for each steroid. These normalized data could be described by a simplified version of eq 1:

$$\Delta Q = \frac{a}{1 + \exp\frac{(T - T_m)}{dT}} \qquad (2)$$

where a is the upper asymptote. Note that ΔQ (or ΔP) in eq 2 approaches a when the temperature is low (i.e., high domain extent or L_o phase) whereas it tends to zero when the temperature is high (i.e., low domain extent or L_d phase). The model (eq 2) was then adjusted to the ΔQ and ΔP values following a nonlinear regression procedure whereby all parameters were obtained.

Upon addition of an exogenous steroid, at least two significant changes occur in the shape of the ΔQ and ΔP curves. At low temperatures, a domain-disrupting compound may decrease the fraction of the total bilayer area occupied by the already existing DPPC domains. In the proposed model this fraction is represented by Δa (Figure 3), i.e., the difference between the upper asymptote of a control curve and the experimental curve in the presence of a given steroid, and should be indicative of a decrease in the number and/or the area of DPPC-rich domains. Since a decrease in ΔQ corresponds to a higher amount of quencher in DPPC-rich domains, Δa may also report on the proportion of DPPC molecules relative to those of the quencher in the domain. It should be noted that using the current methodology it is not possible to unequivocally ascertain whether these three phenomena contribute equally to the decrease in Δa or whether any one prevails over the other. The other change observed in the shape of the curves is the shift of the transition temperature (ΔT_m). Our current interpretation of this phenomenon is that domain-inhibiting compounds may decrease the thermal stability of the already existing DPPC domains (Figure 3). It follows from this analysis that the ability of compounds to decrease the extent of the already existing DPPC domains and/or reduce their thermal stability can be determined from the changes in Δa and ΔT_m, respectively.

Effects of Steroids on Lipid Domain Formation/Stabilization. Figure 4 (top half) illustrates the temperature dependence of the normalized experimental data collected from the quenching experiments (ΔQ) and the adjusted Boltzman-

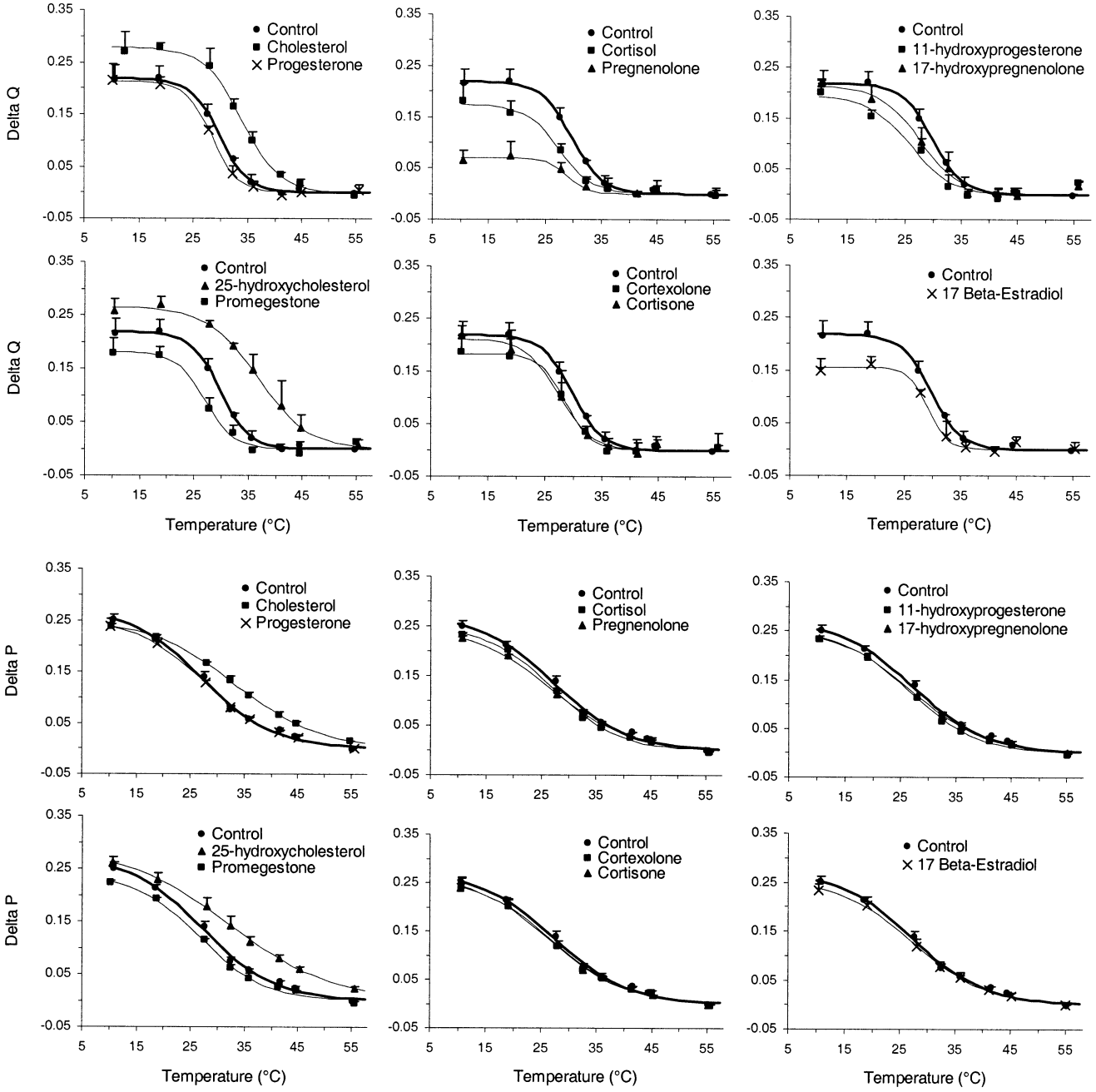

FIGURE 4: Top panels: Difference between quenching values at the working temperature and at the highest temperature (ΔQ) calculated from the experimental fluorescence quenching data (symbols) and fitted with the model described in eq 2 (lines) used to assess the ability of steroids to promote/disrupt lipid domain formation/stabilization. The model was adjusted using mean values of ΔQ (see text for details). Bottom panels: Difference between polarization values at the working temperature and values at the highest temperature (ΔP) calculated from the experimental fluorescence polarization data (symbols) and fitted with the model described in eq 2 (lines). The model was adjusted using mean values of ΔP (see text for details). For reasons of clarity, in both panels results are shown in six separated plots with the control sample in each one. Error bars represent the standard deviation of at least three independent experiments.

type equation (eq 2) for various steroids. Only Chol and 25-hydroxycholesterol (25-OH-Chol) displayed higher ΔQ values than those of control liposomes at all temperatures. This indicates a simultaneous increase in the total area occupied by the DPPC-enriched domains and in the thermal stability of such domains. Other steroids showed lower ΔQ values than those of control liposomes, thus indicating a simultaneous decrease in the total area occupied by DPPC domains and in their thermal stability. Differences in the shape of the curve are observed among different steroids (see, e.g., pregnenolone), with a varying decrease in Δa and/or in ΔT_m. This will be further discussed below.

Steady-state DPH polarization has been widely used since early days (*27*) to study the phase transition of phospholipid bilayers because of the drastic changes in its fluorescence polarization occurring at, or close to, the transition temperature. Lipids in the L_o phase display a relatively fast lateral diffusion but an ordered two-dimensional disposition (*17*). In such lipid environment, DPH molecules also exhibit a high degree of order and a restricted rotational reorientation, yielding high values of fluorescence polarization. Mixtures of DPPC/DOPC/Chol or DPPC/DOPC/25-OH-Chol were the only ones to exhibit higher ΔP values than control MLV (Figure 4, bottom panel). Other steroids exhibited lower ΔP

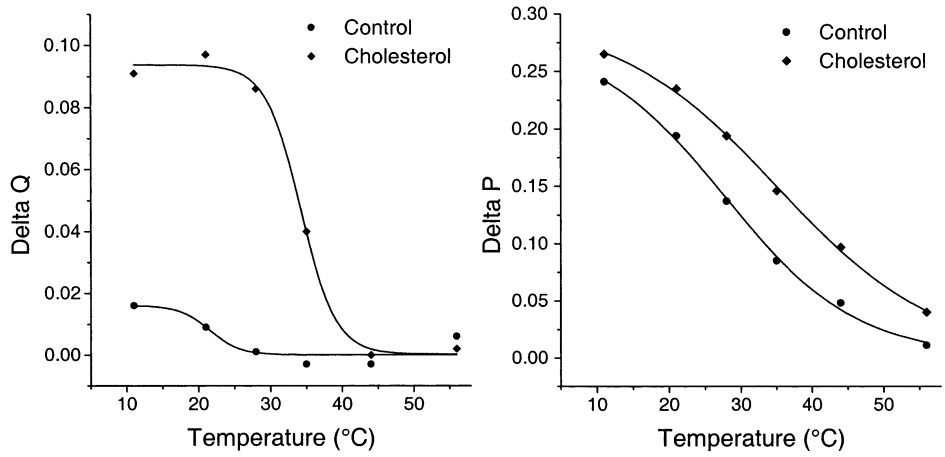

FIGURE 5: Difference between the quenching (left, ΔQ) and polarization (right, ΔP) values at the working temperature and the values at the highest temperature, used to assess the ability of Chol to promote lipid domain formation in Sph/Chol liposomes. Experimental fluorescence quenching data were fitted with the model described in eq 2.

values. Polarization was also measured in all liposomes listed in Table 1, with and without added steroids. In all of these different lipid/quencher mixtures, steroids showed the same trend: Chol- and 25-OH-Chol-containing liposomes were the only ones showing ΔP values higher than those of the control samples having no steroid (data not shown).

Chol Also Promotes Lipid Domain Formation in Sph/DOPC/Chol Liposomes. To ascertain if the observed domain-promoting activity of the sterol Chol in the DPPC/DOPC/Chol system is also exerted in a system containing Sph, we performed a series of experiments using Sph instead of DPPC. Figure 5 shows the temperature dependence of ΔQ and ΔP in Sph/DOPC/Chol liposomes. The sigmoidal shape of the curves is similar to that of the reference system containing DPPC instead of Sph (Figure 4). In the presence of the sterol, Chol, the values of ΔP, and in particular ΔQ, are higher than those of control curves at any temperature. The thermal dependence of ΔQ and ΔP for the Sph-containing system is consistent with the hypothesis that, in simple ternary systems mimicking the elementary composition of the so-called lipid rafts, Chol promotes the formation of a liquid-ordered phase (*2*, *15*−*20*).

Domain Formation/Stabilization Coefficient (DFSC). A steroid can change the total extent of DPPC-rich domains (i.e., change Δa) and/or their thermal stability (i.e., change ΔT_m). To better assess the lipid domain-promoting or -disrupting ability of the steroids, we introduced an algorithm that we coined the domain formation/stabilization coefficient (DFSC):

$$\mathrm{DFSC} = \frac{\Delta a}{a} + \frac{\Delta T_m}{T_m} \qquad (3)$$

where $\Delta a/a$ is the lipid domain formation parameter, indicating the total domain extent with respect to the control sample, and $\Delta T_m/T_m$ is the stabilization parameter, which reflects the thermal stability of the already existing domain with respect to the control sample (Figure 3). DFSC is defined such that, for example, it is null when the weight fraction of each term in the equation is equal and opposite in sign or when both are equal to zero. Thus, a steroid that diminishes both the extent and the stability of DPPC domains will yield a negative value of DFSC and vice versa. A steroid that increases the extent but not the thermal stability of the lipid domain can have a DFSC near zero (i.e., near the DFSC of the control sample). The values obtained for DFSC and their two terms, calculated from the quenching and polarization experimental data, are shown in Figure 6 and Table 2, respectively.

All steroids, except for Chol and 25-OH-Chol, showed negative DFSC values using any of the two sets of data, thus indicating an integral DPPC-rich domain-disrupting activity. The analysis of the domain formation and stabilization parameters calculated from the quenching experiments indicates that all of these steroids showed negative values (Table 2). In the polarization experiments, the domain formation parameters were also negative, and only the stabilization parameters were slightly positive in the case of progesterone and 17β-estradiol. Chol and 25-OH-Chol showed positive values in their domain formation and stabilization parameters. Chol also showed a slightly negative value for the formation parameter (−0.09) calculated from fluorescence polarization data. This series of experiments suggests that steroids that reduce (or expand) the total area occupied by DPPC-rich domains also decrease (or increase) their thermal stability, albeit not necessarily to the same extent. These results are not totally unexpected, since compounds that promote the interaction between DPPC molecules and increase their bilayer fractional area also tighten and stabilize the already existing lipid domains.

From comparison of ΔQ and ΔP curves (Figure 4) and DFSC parameters (Table 2), fluorescence polarization appears to be less sensitive for the detection of lipid domain formation and/or stabilization than is fluorescence quenching. The sigmoid-shaped curves of polarization data for each steroid are closer to the control sample than those corresponding to the quenching data. However, the fact that lipid domain-promoting (e.g., Chol and 25-OH-Chol) or domain-disrupting (the rest) steroids either increase or decrease both ΔQ and ΔP values points to the overall correlation between the two fluorescence methodologies employed in their ability to report on the occurrence of lipid domains. This is reinforced by the high correlation coefficient (r) between DFSC quenching and polarization values (inset to Figure 6). When a given steroid increases the fractional area of DPPC-rich domains and the amount of lipids in the tight L_o phase

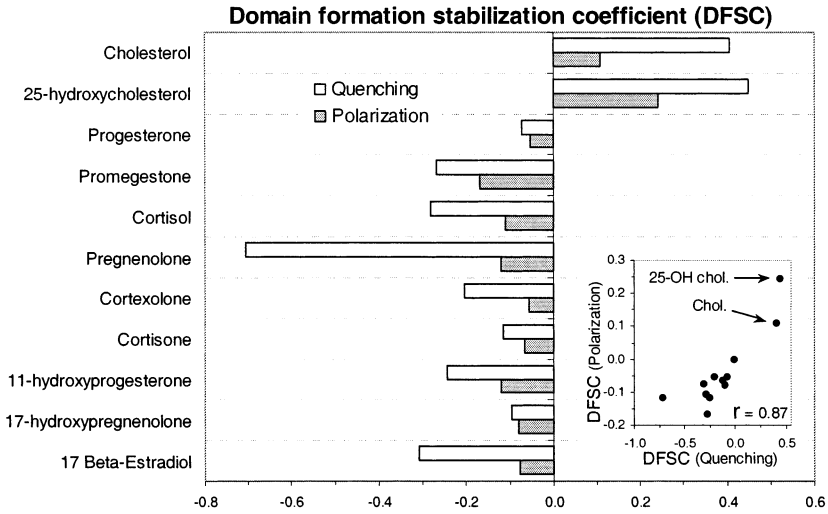

FIGURE 6: Domain formation/stabilization coefficient (eq 3) reflecting the integral DPPC-rich domain-promoting (positive values) or -disrupting (negative values) activity of steroids (see text for details). Inset: Cluster analysis showing the correlation between calculated DFSCs from the experimental fluorescence quenching and polarization data, respectively.

Table 2: Parameters Derived from Fluorescence Quenching and Polarization Experiments (Eq 2) and DFSC Function (Eq 3) To Quantify the Propensity of Steroids To Form and Stabilize or, Alternatively, To Disrupt and Destabilize Lipid Domains

	quenching					polarization				
	parameters in eq 2			DFSC parameters (eq 3)		parameters in eq 2			DFSC parameters (eq 3)	
steroid	a	T_m	dT	formation ($\Delta a/a$)	stabilization ($\Delta T_m/T_m$)	a	T_m	dT	formation ($\Delta a/a$)	stabilization ($\Delta T_m/T_m$)
control	0.22	29.8	2.7	0	0	0.27	27.2	6.6	0	0
Chol	0.28	33.9	3.4	0.27	0.138	0.25	33.3	7.9	−0.09	0.202
25-OH-Chol	0.27	36.8	4.6	0.21	0.235	0.29	32.0	9.7	0.08	0.161
progesterone	0.21	28.4	2.4	−0.03	−0.046	0.26	27.8	6.8	−0.07	0.018
promegestone	0.18	27.0	2.6	−0.17	−0.093	0.24	26.9	6.0	−0.16	−0.013
cortisol	0.17	27.4	3.0	−0.20	−0.079	0.25	27.1	6.0	−0.10	−0.004
pregnenolone	0.07	29.0	1.8	−0.68	−0.025	0.25	26.5	7.2	−0.10	−0.024
cortexolone	0.18	28.6	2.2	−0.16	−0.041	0.27	26.5	6.8	−0.03	−0.025
cortisone	0.21	27.4	2.9	−0.04	−0.079	0.26	27.2	6.6	−0.06	−0.002
11-hydroxyprogesterone	0.19	25.9	3.6	−0.11	−0.131	0.25	26.9	6.1	−0.11	−0.012
17-hydroxypregnenolone	0.21	27.6	3.5	−0.02	−0.073	0.26	26.9	6.9	−0.07	−0.012
17β-estradiol	0.16	29.3	1.7	−0.29	−0.018	0.25	27.4	6.6	−0.08	0.007

(i.e., increased ΔQ), a reduction in the motion of DPH molecules results, yielding high fluorescence polarization values. On the other hand, a domain-disrupting steroid decreases the extent of DPPC-rich domains and decreases the rigidity of (a large fraction of) the bilayer, thus resulting in low fluorescence polarization values.

Relationship between Steroid Hydrophobicity and Steroid Effects on Lipid Domains. The partition coefficient of a compound in octanol/water is considered useful for indirectly assessing its partition behavior in a lipid bilayer/water system (*28*). To determine whether there is a relationship between the hydrophobicity of steroids and their lipid domain-promoting or -disrupting activity, the octanol/water partition coefficient of steroids was compiled from the literature (*29*−*35*) and also calculated using the Log P software (*36*). The latter is based on an algorithm that splits the molecule into small structural units of known Log P and then predicts the value for the entire molecule. As the values obtained from these two sources (i.e., literature and calculation) were very similar to each other, a mean Log P value was calculated. Analogously, the positive correlation between quenching and polarization results suggested the use of a single parameter calculated from ΔQ and ΔP experimental data to assess the overall lipid domain "activity" of a given steroid [i.e., the DFCS coefficient (eq 3)]. Figure 7 shows the relationship between the mean steroid hydrophobicity and mean DFSC values. It can be observed that the lipid domain-disrupting steroids are all clustered at low Log P and DFSC values, whereas Chol and 25-OH-Chol are separated into a cluster exhibiting the highest Log P and DFSC values. None of the assayed steroids had intermediate Log P values (i.e., 4.5−7.5). To compare the findings of London and co-workers (*1*, *2*) on some lipid domain-promoting or -disrupting steroids with ours, the model (eq 2) was adjusted to the experimental data, and DFSC was calculated according to eq 3 (Figure 7). Steroids that Xu et al. (*2*) interpreted as lipid domain-promoting, including Chol and 25-OH-Chol, yielded positive DFSC values with respect to the control samples and exhibited high Log P values which also bore similarity to each other. Conversely, some of the lipid domain-disrupting steroids studied by London's group (*1*, *2*) exhibited low Log P values (e.g., androstenol) whereas others (C4 sterol and cholestenone) exhibited relatively high Log P values.

Although structural and physicochemical factors other than hydrophobicity may influence the effect of steroid on DPPC domains, the cluster analysis used here suggests that the

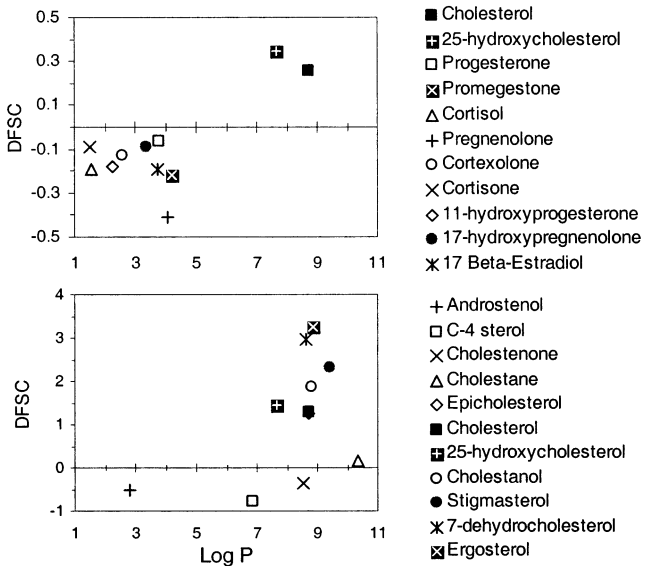

FIGURE 7: Upper panel: Relationship between the mean Log P of steroids (obtained from literature data and numerical calculation) and the mean DFSC, calculated from the fluorescence quenching and polarization data (this work). Lower panel: DFSC values calculated from fluorescence quenching experimental data from refs *1* and *2*.

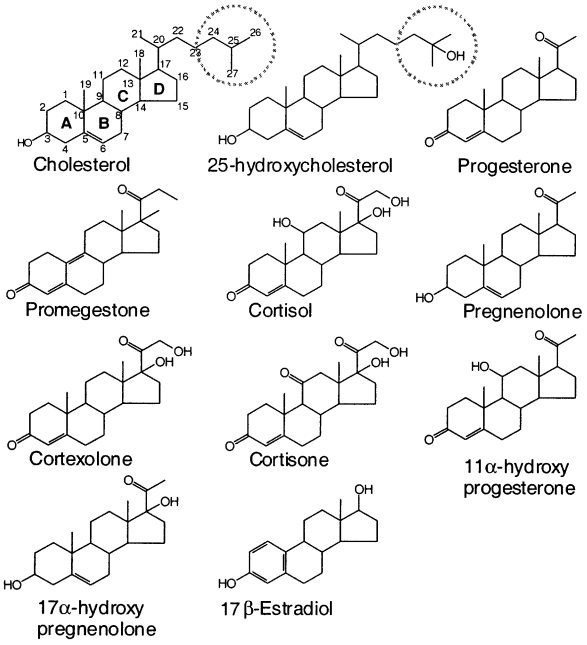

FIGURE 8: Structure of the steroids studied in the present work. Circles outline the main structural traits in the lipid domain-promoting steroids.

higher the hydrophobicity of the steroid, the stronger the integral DPPC-rich domain-promoting activity, and vice versa.

Relationship between Steroid Structure and Sterol Effect on Lipid Domains. Figure 8 shows the structural formulas of the steroids studied here. It can be observed that Chol and 25-OH-Chol are the only two compounds in the series that possess an isooctyl side chain bond at C17. The carbons attached to C23 (i.e., C24−C27) are not present in any of the other steroids. Thus, this feature constitutes the main structural difference between lipid domain-promoting and -disrupting steroids. It is interesting to correlate our findings on the influence of the aliphatic side chain with the characterization of androstenol and C4 sterol by Xu and London (*1*) and Xu et al. (*2*). These steroids display lipid domain-disrupting activity and are the only ones having no carbon side chain, which is in fact present in domain-promoting steroids such as Chol and 25-OH-Chol. According to this and to our calculated DFSC values (Figure 7), androstenol and C4 sterol exhibit a domain-disrupting activity, and neither has an aliphatic side chain. The synthetic C4 sterol is almost identical to Chol, except that its carbon chain is shortened at C23, with four carbons less than Chol. Androstenol lacks the entire side chain; it has eight carbons less than Chol and contains instead a polar hydroxyl group attached to C17. An exception is cholestenone, which possesses a carbon side chain identical to Chol. The fact that a Chol-like tail is absent in almost all lipid domain-disrupting steroids is consistent with models envisaging transbilayer tail-to-tail dimer organization of Chol and other steroids in membranes (*37*, *38*).

It is noteworthy that addition of an aliphatic side chain in itself increases the hydrophobicity of any molecule and that the larger the number of aliphatic carbons, the higher the Log P value (*28*). Hence, the high hydrophobicity of lipid domain-promoting steroids strongly indicates that, for steroid-like compounds, a carbon side chain at least as long as that present in Chol is necessary for promoting lipid domain formation. Since steroids anchor in the bilayer with their 3-hydroxyl group at the level of the polar phosphate headgroup region (*39*), the hydrophobicity conferred by this side chain to the opposite end of the molecule would help in anchoring it in the bilayer. This could lead to a better alignment, a high phospholipid−phospholipid and phospholipid−steroid interaction, and tight packing, promoting saturated lipid domain formation and stabilization. The steroids studied in this work also exhibit differences among their substituent groups at positions C3, C10, C11, C17, and C20, in the presence or absence of double bonds between C4−C5, C5−C6, and C9−C10, and in the A ring (Figure 8). 17β-Estradiol is in fact the only steroid in the series studied here that possesses an aromatic A ring.

The presence of a polar group (i.e., hydroxyl or keto) in the flat fused ring and/or C20 is a common structural feature in several lipid domain-disrupting steroids (see Figure 8). A polar substituent positioned inside the hydrophobic region of the bilayer (except for those in C3, located near the polar headgroup region) would certainly destabilize the packing of lipid domains. It should be noted that a saturated carbon chain has a higher affinity for Chol (and other steroids lacking polar groups positioned in the hydrophobic core) than unsaturated lipids, and these steroids tend in general to stiffen chain ends but lower the average chain order near the lipid headgroup (*40*). These steroids also display drying effects on bilayers constituted by either saturated or unsaturated lipids, removing water and promoting a close contact and van der Waals interaction between adjacent lipid molecules (*24*, *41*). On the contrary, those steroids having polar groups at a bilayer depth equivalent to that of the acyl chains can be expected to disrupt the ordered packing of phospholipids and were in fact found to be lipid domain-disrupting steroids in the present work.

The polar groups attached to the flat fused rings at positions different from C3 may not be the only lipid domain-

disrupting feature of such groups; the steric constraints caused by such groups could additionally act as a factor disturbing lipid packing. A recent study using synthetic membranes (*42*) shows that lanosterol, which is similar to Chol but has three extra methyl groups bound to the flat fused rings (two at position C5 and one at position C14), decreases lipid order and the degree of packing of saturated lipids. Thus, a smooth and streamlined molecular structure at the steroid hydrophobic moiety, like that present in Chol, may contribute to a strong interaction with lipid acyl chains.

The preferential interaction and packing of Chol and similar steroids with membrane phospholipids do not occur through contact with acyl chains only; interactions also involve lipid headgroups (*24*, *43*). Thus, different matrix phospholipids with similar acyl chains affect Chol desorption from lipid vesicles and exposure of Chol to oxidation by Chol oxidase to different extents (*44*). Among the common membrane lipids, Sph is preferentially selected by Chol. This may explain, at least in part, why Sph is usually associated with Chol in the so-called detergent-resistant membranes and lipid rafts.

The lipid domain-promoting and -disrupting steroids studied in the present work have a polar group at position C3, which allows the molecule to orient with this region toward the phospholipid polar headgroup. The structure of cholestanol and cholestane is very similar to that of Chol and 25-OH-Chol (including the aliphatic side chain), the only difference between the two being that cholestanol has a hydroxyl group at position −3 whereas cholestane has none. Both steroids display lipid domain-promoting activity, which is higher in the case of cholestanol (Figure 7). This finding and the fact that a polar group at position C3 also hinders steroid flip-flop from one leaflet to the other by its hydrogen binding ability (*45*) suggest that the presence of a polar substituent in such position affects both the anchoring and orientation of the molecule in the bilayer.

Our findings on the influence of the above-mentioned structural characteristics of steroids indicate that lipid domain-promoting activity is likely to be associated to their ability to strengthen close packing with saturated lipids; conversely, those compounds that impair lipid packing behave as domain-disrupting compounds. Interestingly, the structural features of lipid domain-promoting steroids correspond to those of the so-called "membrane-active steroids" (*24*), a term used in reference to steroids known to modulate membrane properties such as barrier properties and other physicochemical characteristics of lipid bilayers. The minimal structural requirements for these membrane-active steroids include a flat and rigid fused ring system, a small polar group at position C3, a Chol-like tail, and a relatively small area (around 40 $Å^2$/molecule) at the water interface.

Chol and other neutral lipids are important for the function of some cell surface receptors, such as the nicotinic acetylcholine receptor (see reviews in refs *46* and *47*). The possible modulation of acetylcholine receptor structure and/or function by sterols was postulated in early electron spin resonance experiments in which the presence of sterols in the receptor-vicinal lipid could be demonstrated (*48*, *49*). Patch-clamp electrophysiological studies at the single channel level established the modulatory role exerted by several steroids, some of which have been shown to be lipid domain-promoting sterols, on receptor function (*29*, *50*−*53*). Future work will be aimed at investigating the interaction of lipid domain-promoting or -disrupting steroids with the acetylcholine receptor protein using the techniques employed here.

ACKNOWLEDGMENT

We thank Prof. M. Prieto for valuable discussions.

REFERENCES

1. Xu, X., and London, E. (2000) *Biochemistry 39*, 843−849.
2. Xu, X., Bittman, R., Duportail, G., Heissler, D., Vilchezes, C., and London, E. (2001) *J. Biol. Chem. 276*, 33540−33546.
3. Brown, D., and Rose, J. K. (1992) *Cell 68*, 533−544.
4. Simons, K., and Ikonen, E. (1997) *Nature 387*, 569−572.
5. Thompson, T. E., and Tillack, T. W. (1985) *Annu. Rev. Biophys. Biophys. Chem. 14*, 361−386.
6. Rietveld, A., and Simons, K. (1998) *Biochim. Biophys. Acta 1376*, 467−479.
7. Brown, D. A., and London, E. (1998) *Annu. Rev. Cell Dev. Biol. 14*, 111−136.
8. Simons, K., and Ikonen, E. (2000) *Science 290*, 1721−1726.
9. Heino, S., Lusa, S., Somerharju, P., Ehnholm, C., Olkkonen, V. M., and Ikonen, E. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 8375−8380.
10. Herreros, J. N., and Schiavo, G. (2001) *Mol. Biol. Cell 12*, 2947−2960.
11. Nguyen, D. H., and Taub, D. (2002) *J. Immunol. 168*, 4121−4126.
12. Popik, W., Alce, T. M., and Au, W. C. (2002) *J. Virol. 76*, 4709−4722.
13. Anderson, R. G. W., and Jacobson, K. (2002) *Science 296*, 1821−1825.
14. Schroeder, R., London, E., and Brown, D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12130−12134.
15. Schroeder, R. J., Ahmed, S. N., Zhu, Y., London, E., and Brown, D. A. (1998) *J. Biol. Chem. 273*, 1150−1157.
16. Ahmed, S. N., Brown, D. A., and London, E. (1997) *Biochemistry 36*, 10944−10953.
17. Ipsen, J. H., Karlstrom, G., Mouritsen, O. G., Wennerstron, H., and Zuckermann, M. J. (1987) *Biochim. Biophys. Acta 905*, 162−172.
18. Vist, M. R., and Davis, J. H. (1990) *Biochemistry 29*, 451−464.
19. Sankaram, M. B., and Thompson, T. E. (1990) *Biochemistry 29*, 10670−10675.
20. Feigenson, G., and Buboltz, J. (2001) *Biophys. J. 80*, 2775−2788.
21. London, E., and Brown, D. A. (2000) *Biochim. Biophys. Acta 1508*, 182−195.
22. Sot, J., Collado, I., Arrondo, J. L. R., Alonso, A., and Goñi, F. M. (2002) *Langmuir 18*, 2828−2835.
23. Heerklotz, H. (2002) *Biophys. J. 83*, 2693−2701.
24. Barenholz, Y. (2002) *Lipid Res. 41*, 1−5.
25. Friedrichson, T., and Kurzchalia, T. V. (1998) *Nature 394*, 802−805.
26. Varma, R., and Mayor, S. (1998) *Nature 394*, 798−801.
27. Lentz, B. R., Barenholz, Y., and Thompson, T. E. (1976) *Biochemistry 15*, 4529−4537.
28. Leo, A., Jow, P. Y., Silipo, C., and Hansch, C. (1975) *J. Med. Chem. 18*, 865−868.
29. Garbus, I., Bouzat, C., and Barrantes, F. J. (2001) *NeuroReport 12*, 227−231.
30. Valvani, S. C., and Yalkowsky, S. H. (1980) in *Physical Chemical Properties of Drugs* (Yalkowsky, S. H., Sinkula, A., and Valvani, S., Eds.) Marcel Dekker, New York.
31. Yalkowsky, S. H., Valvani, S. C., and Roseman, T. J. (1983) *J. Pharm. Sci. 72*, 866−870.
32. Ponec, M., Kempenaar, J., Shroot, B., and Caron, J. C. (1986) *J. Pharm. Sci. 75*, 973−975.
33. Pinsuwan, S., Li, A., and Yalkowsky, S. H. (1995) *J. Chem. Eng. Data 40*, 623−626.
34. Alvarez Núñez, F. A., and Yalkowsky, S. H. (1997) *J. Pharm. Sci. 86*, 1187−1189.
35. SRC Interactive PhysProp Database (2002) Synopsys Scientific Systems, Ltd., http://esc.syrres.com/interkow/physdemo.html.
36. KowWin (LowKow) for Microsofts Windows Version 1.65 (1999) Syracuse Research Corp. (SRC), New York.
37. Harris, J. S., Epps D. E., Davio S. R., and Kezdy, F. J. (1995) *Biochemistry 34*, 3851−3857.

38. Loura, L. M., and Prieto, M. (1997) *Biophys. J. 72*, 2226−2236.
39. Worcester, D. L., and Franks, N. P. (1976) *J. Mol. Biol. 100*, 359−378.
40. Mitchell, D. C., and Litman, B. J. (1998) *Biophys. J. 75*, 896−908.
41. Straume, M., and Litman, B. J. (1987) *Biochemistry 26*, 5121−5126.
42. Miao, L., Nielsen, M., Thewalt, J., Ipsen, J. H., Bloom, M., Zuckermann, M. J., and Mouritsen, O. G. (2002) *Biophys. J. 82*, 1429−1444.
43. Samsonov, A. V., Mihalyov, I., and Cohen, F. S. (2001) *Biophys. J. 81*, 1486−1500.
44. Samuni, A. M., Lipman, A., and Barenholz, Y. (2000) *Chem. Phys. Lipids 105*, 115−119.
45. Addona, G. H., Sandermann, H., Kloczewiak, M. A., Husain, S. S., and Miller, K. W. (1998) *Biochim. Biophys. Acta 1370*, 299−309.
46. Barrantes, F. J. (1993) *FASEB J. 7*, 1460−1467.
47. Barrantes, F. J. (2003) *Curr. Opin. Drug Discov. Dev. 6*, 620−632.
48. Marsh, D., and Barrantes, F. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4329−4333.
49. Marsh, D., Watts, A., and Barrantes, F. J. (1981) *Biochim. Biophys. Acta 645*, 97−101.
50. Bouzat, C., and Barrantes, F. J. (1993) *NeuroReport 4*, 143−146.
51. Bouzat, C., and Barrantes, F. J. (1993) *Mol. Neuropharmacol. 3*, 109−116.
52. Bouzat, C., and Barrantes, F. J. (1996) *J. Biol. Chem. 271*, 25835−25841.
53. Garbus, I., Roccamo, A. M., and Barrantes, F. J. (2002) *Neuropharmacology 43*, 65−73.

BI035759C