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Dipole Potential and Head-Group Spacing Are Determinants for the Membrane Partitioning of Pregnanolone

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ABSTRACT

The membrane interactions of pregnanolone, an intravenous general anesthetic steroid, were characterized using fluorescence spectroscopy and monolayer technique. di-8-ANEPPS [4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3- sulfopropyl)-pyridinium], a membrane dipole potential (Ψ)-sensitive probe, revealed pregnanolone to decrease Ψ similarly as reported previously for other anesthetics. The decrement in Ψ was approximately 16 and 10 mV in dipalmitoylphosphatidylcholine (DPPC) and DPPC/cholesterol (90:10, mol/mol) vesicles, respectively. Diphenylhexatriene anisotropy indicated pregnanolone to have a negligible effect on the acyl chain order. In contrast, substantial changes were observed for the fluorescent dye Prodan, thus suggesting pregnanolone to reside in the interfacial region of lipid bilayers. Langmuir balance studies indicated increased association of pregnanolone to DPPC monolayers containing cholesterol or 6-ketocholestanol at surface pressures $\pi > 20$ mN/m as well as to monolayers of the unsaturated 1-palmitoyl-2-oleoylphosphatidylcholine. In the same surface pressure range, the addition of phloretin, which decreases Ψ, reduced the penetration of pregnanolone into the monolayers. These results suggest that membrane partitioning of pregnanolone is influenced by the spacing of the phosphocholine head groups as well as by membrane dipole potential. The latter can be explained in terms of electrostatic dipole-dipole interactions between pregnanolone and the membrane lipids with their associated water molecules. Considering the universal nature of these interactions, they are likely to affect membrane partitioning of most, if not all, weakly amphiphilic drugs.

Pregnanolone (Fig. 1) is a steroid-based progesterone metabolite that is used as an intravenous general anesthetic (Hering et al., 1996). Because of its low solubility into water, it is administered in an oil-in-water emulsion (eltanolone). Hydrophobicity of compounds is directly linked to their membrane partitioning, and thus, pregnanolone can be expected to favor association with lipid bilayers. However, as far as we are aware, no studies on pregnanolone-lipid interactions have been reported. Other general anesthetics such as isoflurane and halothane have been shown to interact with lipid membranes (Cafiso, 1998). Although many different modes of action have been suggested, the exact mechanism of action of general anesthetics has remained unresolved. The proposed mechanisms include interactions between anesthetics and proteins, interactions between anesthetics and lipids, and effects of anesthetics on the lipid-protein interface (Makriyannis et al., 1990; Ueda et al., 1994). Cantor (1997) suggested a mechanism linking the membrane lateral-pressure profile to the action of membrane proteins. The importance of nonspecific interactions mediated by the amino acid residues in the lipid-water-protein interface was highlighted in a recent molecular-dynamics simulation of the effects of an anesthetic on gramicidin channels in membranes (Tang and Xu, 2002).

The nongenomic effects of steroids, such as anesthesia, require higher steroid concentrations to manifest themselves than those mediated by the nuclear steroid receptors (Duval et al., 1983). Although these nongenomic effects are well-documented, their mechanisms are still in dispute, and a number of molecular-level events could be involved. In this respect, efforts have been undertaken to find specific membrane steroid receptors (Wehling, 1997). Regarding anes-

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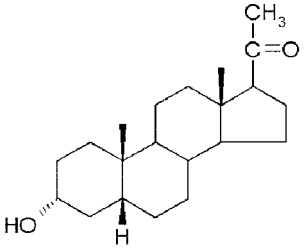
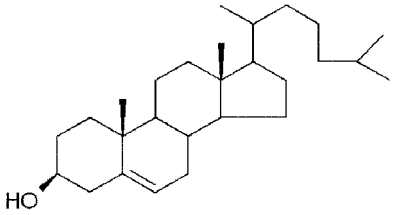
ABBREVIATIONS: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; di-8-ANEPPS, 4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl)-pyridinium; DPH, 1,6-diphenyl-1,3,5-hexatriene; GP, generalized polarization; LUV, large unilamellar vesicle; mD, milliDebye; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DMSO, dimethyl sulfoxide; Prodan, 6-propionyl-2-dimethylaminonaphthalene; r, fluorescence anisotropy; X_A, mole fraction of substance A; π, monolayer surface pressure; Ψ, membrane dipole potential.

thetic activity, possibilities remaining under consideration are the binding of steroids to membrane receptors such as GABA receptor (Prince and Simmons, 1993; Wehling, 1997) and indirect effects mediated via the membrane lipid matrix (Makriyannis et al., 1990; Ueda et al., 1994) or the protein-water interface (Ueda et al., 1994). Not surprisingly, there is no correlation between hormonal and anesthetic activities of the steroids, such as pregnanes, androgens, and corticosteroids (Duval et al., 1983). Anesthetic efficiency, in general, correlates well with the partitioning of the drug into the membrane-water interface (Ueda and Yoshida, 1999). As for other groups of anesthetics, the efficiencies of derivatives of a steroid compound, alphaxalone, correlated reasonably well with the extent of membrane perturbation detected by NMR (Makriyannis et al., 1990, 1991; Ueda et al., 1994).

The lipid membrane-mediated mechanism of action of general anesthetics would not be unique, because for some drugs [e.g., amphotericin B (Bolard, 1986)], the lipid membrane has been shown to be the target, thus involving no protein receptors. In addition, cardiotoxicity of doxorubicin (Goormaghtigh et al., 1982) and pulmonary toxicity of amiodarone (Reasor and Kacew, 1996) are proposed to be consequences of direct drug-lipid interaction, leading to the deterioration of the physiological functions of certain proteins. Biomembranes are known to be laterally heterogeneous in lipid and protein distribution (Kinnunen 1991; Mouritsen and Kinnunen, 1996). We have proposed that both therapeutic and adverse effects of drugs could be related to their ability to modulate membrane dynamics and lateral organization as well as the membrane association of peripheral proteins (Kinnunen, 1991; Söderlund et al., 1999; Jutila et al., 1998, 2001). The importance of membrane lateral organization is reflected in the membrane association and function of some proteins (e.g., cytochrome *c*) and processes, such as the sorting of proteins in intracellular transport. To date, several mechanisms contributing to the membrane dynamics and lateral organization have been described previously (Kinnunen 1991; Mouritsen and Kinnunen, 1996; Holopainen et al., 2004).



pregnanolone



cholesterol

Fig. 1. Chemical structure of pregnanolone. The structure of cholesterol is shown for comparison.

The main emphasis of this study was to investigate the membrane properties affecting partitioning of pregnanolone as well as to characterize the effect of pregnanolone on the membrane dynamics and organization. Lateral membrane domains with different lipid compositions are present in biological membranes (Kinnunen, 1991). Although drugs may modulate the lateral organization and composition of membrane microdomains (Söderlund et al., 1999b; Jutila et al., 2001), the great variation in the membrane association for different lipid compositions suggests that the membrane-bound compounds could be present at very different local concentrations in these membrane domains.

Materials and Methods

Materials. HEPES, EDTA, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), phloretin, 6-ketocholestanol, and 5-cholesten-3β-ol (β-cholesterol) were purchased from Sigma Chemical (St. Louis, MO). Dimethyl sulfoxide (DMSO) was from Merck (Darmstadt, Germany). 5β-Pregnan-3β-ol-20-one (pregnanolone) was obtained from Steraloids (Wilton, NH). 6-Propionyl-2-dimethylaminonaphthalene (Prodan) and di-8-ANEPPS were from Molecular Probes (Eugene, OR), and 1,6-diphenyl-1,3,5-hexatriene (DPH) was from EGA Chemie (Steinheim, Germany). Deionized water was filtered through a Millipore filter (Millipore Corporation, Bedford, MA). Purity of the lipids was checked on silicic acid-coated plates (Merck) using a chloroform/methanol/water solution (65:25:4, v/v/v) as a solvent. Examination of the plates after iodine staining or fluorescence illumination revealed no impurities. Lipid stock solutions were made in chloroform. Concentrations of the nonfluorescent lipids were determined gravimetrically using a high-precision Cahn 2000 electrobalance (Cahn Instruments, Inc., Cerritos, CA). Concentrations of the fluorescent probes di-8-ANEPPS, DPH, and Prodan were determined photometrically in methanol, using ϵ = 36 800 $cm^{-1}M^{-1}$ at 498 nm, ϵ = 88,000 $cm^{-1}M^{-1}$ at 350 nm, and ϵ = 18,000 $cm^{-1}M^{-1}$ at 361 nm, respectively.

Preparation of Liposomes. Lipids were mixed to yield the desired molar ratios in chloroform. The mixtures were dried under a stream of nitrogen, and the dry residues were kept under reduced pressure for at least 2 h to remove residual solvent. Samples were hydrated in a buffer (5 mM HEPES and 0.1 mM EDTA, pH 7.4) for 30 min at a temperature of approximately 10°C higher than the main transition temperature to yield multilamellar liposomes. To obtain large unilamellar vesicles (LUVs), the multilamellar liposome dispersions were subsequently extruded through polycarbonate filter (pore size = 0.1 μm; Millipore) using Liposofast-Pneumatic (Avestin, Ottawa, Canada), essentially as described by MacDonald et al. (1991).

Fluorescence Spectroscopy. Fluorescence measurements were carried out with an LS50B spectrofluorometer equipped with magnetically stirred, thermostated cuvette compartment (PerkinElmer Life and Analytical Sciences, Boston, MA). The data were analyzed using the dedicated software provided by PerkinElmer. For the measurement of membrane dipole potential, the fluorescent probe di-8-ANEPPS (Gross et al., 1994) was incorporated (X = 0.01) in DPPC and DPPC/cholesterol LUVs (total lipid concentration, 400 μM). Excitation and emission band-passes were 10 and 5 nm, respectively. Fluorescence emission intensities were measured at 620 nm. The ratio of emission intensities (denoted as R) was obtained using excitation wavelengths of 440 and 530 nm. R has been shown previously to correlate with membrane dipole potential (Gross et al., 1994). The value recorded for DPPC liposomes was used as a reference point for comparison. Phloretin and 6-ketocholestanol were included in liposomes to achieve known changes in membrane dipole potential (Ψ) and were used to obtain the ratio of R versus $\Delta\Psi$ (Fig. 2A), which was used for calibration reference. These $\Delta\Psi$ values were derived from electron paramagnetic resonance measurements of hydrophobic an-

ion and cation binding and translocation rates, and the effective dipole moments were calculated from these values by a model that assumes the additives to be dipoles within the membrane (Franklin and Cafiso, 1993).

DPH (X = 0.002) was incorporated into LUVs to assess the effect of pregnanolone on lipid acyl chain order (Lakowicz, 1999), which correlates with the static fluorescence anisotropy, r, defined as $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$, where $I_{\parallel}$ represents the fluorescence intensity with both polarizers at vertical position, and $I_{\perp}$ represents the intensity with the excitation polarizer at vertical and the emission polarizer at horizontal position. These measurements were conducted using an SLM spectrofluorometer in T-format and equipped with a thermostated cuvette and magnetic stirring. The excitation wavelength was set at 350 nm with a band-pass of 8 nm. On one branch, the emission wavelength of 450 nm was chosen with a monochromator, and the band-pass was set at 16 nm. On the other branch, the emission was selected with an appropriate long-pass filter placed between the sample and the photomultiplier tube.

The polarity-sensitive probe Prodan resides in the interfacial region of the membrane and partitions preferentially into the fluid phase (Krasnowska et al., 1998). Prodan was included into the DPPC LUVs (X = 0.02) to investigate the effect of pregnanolone on the dynamics of the interfacial region of phospholipid bilayer. Generalized polarization (GP) of Prodan has been used previously to measure changes in the microenvironment of the probe, and it is mainly sensitive to water dynamics within the interface (Parasassi et al., 1998), although it is also affected by the partitioning of Prodan between the membrane and the aqueous phase (Krasnowska et al., 1998). Excitation was 350 nm, and GP was calculated from the emission intensities at 425 nm (I_{425}) and 530 nm (I_{530}) by $GP = (I_{425} - I_{530})/(I_{425} + I_{530})$.

The emission spectra of Prodan could be reasonably well-decomposed into two (or three) different Gaussian components at approximately 450 and 505 (and 580) nm. The selected wavelengths 425 and 530 nm correspond to wavelengths at which the first and the second peak had only little overlap.

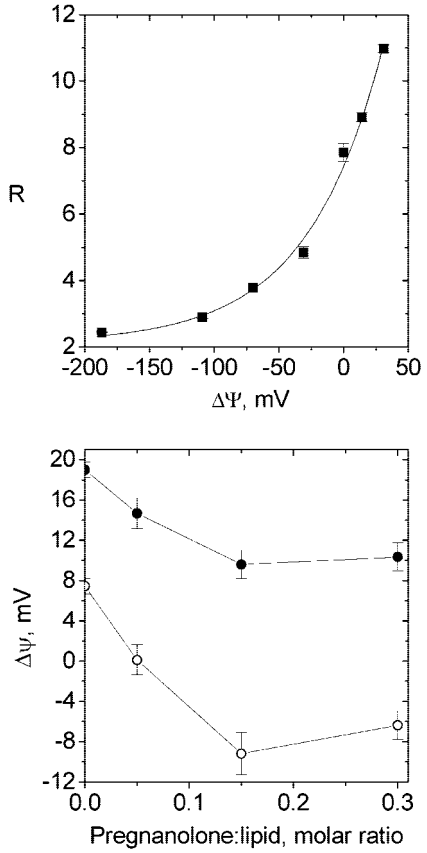


Fig. 2. A, the fluorescence intensity ratio of di-8-ANEPPS as a function of $\Delta\psi$, measured for DPPC/6-ketocholestanol (90:10 and 95:5, mol/mol) and DPPC/phloretin (95:5, 90:10, 85:15, and 75:25) LUVs with the corresponding $\Delta\psi$ values of +31, +14, −31, −70, −109, and −187 mV (D. Cafiso, personal communication). A first-order exponential fit was used with $\chi^2 = 0.12953$. Data points represent the average of three measurements, and error bars show S.D. B, the effects of pregnanolone on the membrane dipole potential in DPPC (■) and DPPC/cholesterol, 90:10 (○) LUVs assessed by di-8-ANEPPS fluorescence. Final lipid concentration was 400 μM in 5 mM HEPES and 0.1 mM EDTA, pH 7.4. Temperature was 45°C. Data points represent the average of three measurements, and error bars show S.D.

Surface Chemistry Measurements. Surface pressure (π) was recorded with a metal alloy wire attached to a microbalance (KBN315; Kibron Inc., Helsinki, Finland) and using magnetically stirred circular wells (1.2 ml, Ø 1 cm). Data were collected and analyzed by dedicated software (FilmWare 2.30) provided by the manufacturer. Phospholipids were dissolved in $CHCl_3$ (final concentration, ~0.5 mM) and were applied onto the air-water interface by a microsyringe. Lipid films were allowed to equilibrate for approximately 10 min to reach stable initial surface pressure (π_0). Thereafter, pregnanolone (dissolved in DMSO) was injected into subphase (5 mM HEPES and 0.1 mM EDTA, pH 7.4) to obtain a final pregnanolone concentration of 2 μM. Penetration of pregnanolone into the lipid film was observed as an increase in π. As a stable π value was reached (within approximately 100 s), it was taken as the final surface pressure, and the difference between this value and π_0 was taken as the increment in surface pressure, $\Delta\pi$ ($\Delta\pi = \pi - \pi_0$). The results are presented as $\Delta\pi$ versus π_0 (Brockman, 1999). Final concentration of DMSO was <1 v-%, which did not affect π. All measurements were done at ≈22°C.

The interfacial area occupied by pregnanolone at the air-water interface was determined by measuring the surface tensions with an eight-channel microtensiometer (δ-8; Kibron Inc.) from a set of solutions with increasing pregnanolone concentrations. For calibration, the first well contained the above buffer with 10 vol-% of the solvent used (DMSO). Surface tension was determined by the du Nouy technique using a small-diameter metal alloy wire probe. Thirteen subsequent wells were measured on each channel in one series. To minimize carryover, the highest drug concentration was in the last sample well. Calculation of the interfacial area occupied by pregnanolone was derived from the Gibbs adsorption isotherm, as described previously (Fischer et al., 1998), with minor modifications. In brief, the adsorption of pregnanolone to the air-water surface decreases the surface tension, γ. The difference between the surface tension of pure water, γ_0, and the new value yields the surface pressure, $\pi = \gamma_0 - \gamma$. Using Gibbs adsorption isotherm, the thermodynamics of this process are given by the equation $d\gamma = -RT\,(N_A A_s)^{-1}\,dlnC = -RT\Gamma dlnC = -d\delta$, where C is the concentration of the amphiphile, RT is the thermal energy, N_A is Avogadro's number, Γ is surface excess concentration, and A_s is the surface area of the amphiphile. By plotting the π versus lnC, a linear slope is obtained (Γ_∞). From these data, A_s is derived using the equation $A_s = (N_A \text{Å}_\infty)^{-1}$. From this, a value of 57 Å^2 was obtained for pregnanolone at the air-water interface.

Compression isotherms were recorded on a trough with two moving barriers (μTroughS; Kibron Inc.), with surface pressure measured as above. The indicated lipid mixtures (dissolved in $CHCl_3$) were applied onto the air-water interface by a microsyringe. The obtained compression isotherms consisting of approximately 800 to 1000 points were smoothed with 11-point adjacent averaging, which had no visible effect on curve shape. The smoothed data set was used for the calculation of isothermal area compressibility κ_T per chain according to the formula

$$\kappa_T = -\frac{1}{A_c}\left(\frac{\partial A_c}{\partial \pi}\right)_T$$

where A_c represents the area per chain. Linear interpolation was used to create data sets of 800 points with the corresponding κ_T and π values, and κ_T was then presented as a function of surface pressure π. With the aid of curves obtained, numerical integration from the initial π_0 (before the addition of pregnanolone into the subphase) to the final π_f (new equilibrium value for π after pregnanolone addition) was carried out to compute the apparent changes in area/chain for the monolayer constituents, ΔA_c:

$$\Delta A_c = [1 - \exp(\int_{\pi_i}^{\pi_f} \kappa_T d\pi)]A_{ci}$$

where A_{ci} is the initial area per chain. The meaning of the obtained ΔA_c value was clarified for viewing by computing the number of pregnanolone molecules penetrating into the monolayer for every 1000 chains using the surface area 57 Å^2. In the calculation of this value, pregnanolone is considered to be a hard, noninteracting particle and to occupy the same area in phospholipid monolayers as on a clean air-water interface. Yet, pregnanolone and phospholipids could very well have preferential interactions that are incompatible with the first assumption, and therefore the number of pregnanolone molecules penetrating into the monolayer would be underestimated. Likewise, if the pregnanolone molecules reside in the phospholipid head-group region as our results suggest, then it is possible that in the vertical profile across the monolayer, there are acyl chains under the cover of pregnanolone and that, accordingly, the effective area occupied by the pregnanolone would be somewhat smaller than the area on the clean air-water interface. Again, the above calculations would slightly underestimate the number of pregnanolone molecules penetrating into the monolayer. These calculations thus give an estimate for the lower limit for the number of pregnanolone molecules associating with the membrane. These data further allow a comparison of the penetration of pregnanolone into monolayers consisting of different lipids. Using this method for different film compositions, it was shown previously that the results on the penetration of estradiol and testosterone into monolayers agree qualitatively with those obtained for steroid association to liposomes assessed by capillary electrophoresis (Wiedmer et al., 2002).

Results

Membrane Dipole Potential Measurements. The presence of pregnanolone decreased Ψ in both DPPC and DPPC/cholesterol LUVs, and at both lipid compositions, saturation was observed at [pregnanolone] = 60 μM, corresponding to a drug/lipid molar ratio of 15:100. For DPPC LUVs, the maximal decrease in Ψ was approximately 16 mV, and for DPPC/cholesterol (90:10, mol/mol) membranes, it was approximately 10 mV (Fig. 2B). These changes are of similar magnitude as reported for some other anesthetics when measured at the minimal alveolar concentration required to induce anesthesia (Qin et al., 1995). Yet, for pregnanolone, minimal alveolar concentration cannot be used because this measure applies only for volatile anesthetics. The reported anesthetic concentration of pregnanolone and those used in vitro and in vivo range from 0.1 to 100 μM (Twyman and MacDonald, 1992; Hering et al., 1996; Edgar et al., 1997).

Effect of Pregnanolone on Membrane Structural Dynamics. To assess the location of pregnanolone in the lipid bilayer, we first measured fluorescence anisotropy r of DPH. However, no changes in this parameter were observed up to the drug/lipid ratio of 3:10 (Fig. 3), thus revealing negligible impact on the acyl chain order of DPPC and DPPC/cholesterol (90:10) LUVs.

Prodan partitions preferentially into the membrane interface (Parasassi et al., 1998) and is thus suitable for investigating the partitioning of a compound into the interfacial region. The GP value for Prodan increases with increasing contents of pregnanolone both above as well as below the transition (Fig. 4), presumably reflecting either a decreased relaxation of water around the excited state of Prodan (Parasassi et al., 1998) or changes in the binding of Prodan to membranes. The latter mechanism could derive from the effects of pregnanolone-induced changes in interfacial dipole density on the energetics of the insertion of Prodan with its associated large dipole moment. In the former case, pregnanolone is likely to make the interface more gel-like and thus restrict the movement of water molecules. Alternatively, pregnanolone could displace water from the interface and decrease the number of water molecules within the immediate vicinity of Prodan. Considering the lack of changes in DPH anisotropy caused by pregnanolone and the presence of the effect on Prodan also below transition, either changes in Prodan partitioning or displacement of water seem plausible; the effects on GP could arise from the partitioning of pregnanolone, water, and Prodan dipoles into the interface, without a significant impact on the molecular order in the interfacial region.

Penetration into Lipid Monolayers. The observed decrease in membrane dipole potential by pregnanolone suggests that membrane dipole potential could influence the interactions of pregnanolone with lipid membranes. Therefore, we investigated the association of pregnanolone with lipid monolayers of different lipid compositions. The latter were selected to have different dipole potentials as well as lateral packing. Increase in the surface pressure ($\Delta\pi$) as a function of lateral packing pressure is shown in Fig. 5. Penetration of pregnanolone into monolayers was rapid, completed within approximately 100 s. For all film compositions studied, $\Delta\pi$ decreased as π_0 was increased. DPPC film was used as a reference. Because the monolayers consisting of different lipids do not have identical area compressibility values, even equal $\Delta\pi$ values for different compositions correspond to nonidentical changes in the area per lipid chain (A_c). To facilitate the comparison for the lipid mixtures studied, we calculated the lower limiting values for the number of pregnanolone molecules penetrating into the membrane for

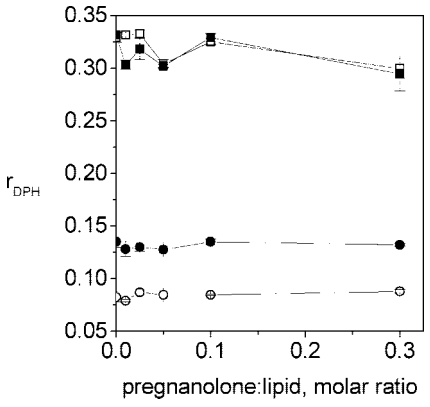


Fig. 3. Fluorescence anisotropy of DPH as a function of pregnanolone content. The effect of pregnanolone on gel (□) and fluid-phase DPPC (○) LUVs as well as gel (■) and fluid-phase DPPC/cholesterol (90:10, ●) LUV was measured. Temperatures were 30°C for gel- and 45°C for fluid-phase liposomes. Final lipid concentration was 50 μM in 5 mM HEPES and 0.1 mM EDTA, pH 7.4.

every 1000 chains, as discussed in under *Materials and Methods*. The values lower than the initial surface pressure of 20 mN/m vary greatly depending on the monolayer composition caused by the phase transitions, extending to these surface pressures. At values greater than π = 20 mN/m, the addition of 6-ketocholestanol (X = 0.20) into DPPC films increased the penetration of pregnanolone (inset in Fig. 6). The opposite effect was seen for phloretin (X = 0.20), with a decrease in the penetration of pregnanolone into the monolayer. These results suggest membrane association of pregnanolone to be dependent on Ψ or on the average dipole moment of monolayer constituents, because phloretin decreases and 6-ketocholestanol increases Ψ (Franklin and Cafiso, 1993). The effect of ΔΨ on the penetration of dipole potential-decreasing membrane association of pregnanolone is thus in accordance with Le Chatelier's principle, in which an increase in Ψ leads to augmented partitioning and a decrease in Ψ leads to attenuated partitioning of a Ψ-decreasing compound. It seems thus logical to assume that the partitioning of pregnanolone into membranes with higher Ψ values is favored by a decrease in dipole repulsion caused by oppositely oriented pregnanolone dipoles partitioning into membranes.

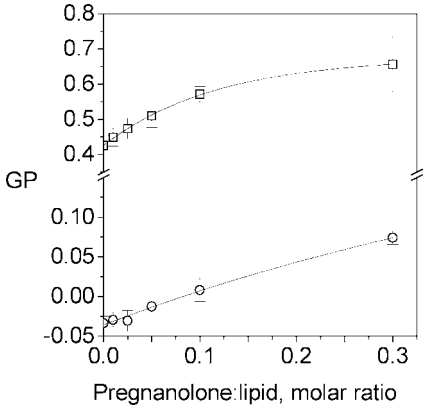


Fig. 4. A, the effect of increasing pregnanolone/lipid ratio on the GP of Prodan in DPPC liposomes at 30 (□) and 50°C (○) fitted with a single exponential decay as a guide for the eye. The data points represent averages of three measurements, and error bars represent standard deviation. The lipid concentration was 50 μM in 5 mM HEPES and 0.1 mM EDTA, pH 7.4.

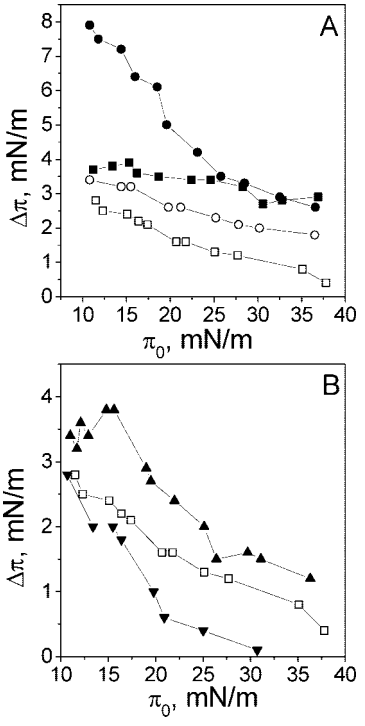


Fig. 5. Penetration of pregnanolone (2 μM) into lipid monolayers observed as an increase in surface pressure ($\Delta\pi$). Lipids were spread in chloroform onto the air buffer (5 mM HEPES and 0.1 mM EDTA, pH 7.4) interface to obtain the indicated initial surface pressure (π_0). A, the effect of POPC and increasing cholesterol content in DPPC monolayers: POPC (○), DPPC (□), DPPC/cholesterol 80:20 (■), and DPPC/cholesterol 60:40 (●) (mol/mol). B, the effect of membrane dipole potential modulating additives on the monolayer penetration of pregnanolone. Monolayer compositions were DPPC/phloretin 80:20 (▼) and DPPC/6-ketocholestanol 80:20 (▲). Penetration of pregnanolone into DPPC (□) is shown for comparison. Temperature was ~22°C.

The presence of cholesterol (DPPC/cholesterol, 80:20, mol/mol) increased pregnanolone partition into monolayers. At the same mole fraction (X = 0.20), 6-ketocholestanol increases the average dipole moment considerably, yet the results show more efficient penetration of pregnanolone in the presence of cholesterol compared with 6-ketocholestanol, thus indicating an additional cholesterol-dependent interaction. An interesting feature was observed when X_{chol} was increased to 0.40. More specifically, the $\Delta\pi$ versus π_0 curve became biphasic, showing an increased penetration at π_0 < 25 mN/m, whereas at higher π_0 values, no difference between X_{chol} = 0.20 and 0.40 was observed. At π = 10 to 25 mN/m, both DPPC/cholesterol mixtures are in the liquid-condensed phase, whereas at X_{chol} = 0.40 the film is more condensed (Smaby et al., 1994). In DPPC monolayers, a liquid-expanded/liquid-condensed phase coexistence region is observed at

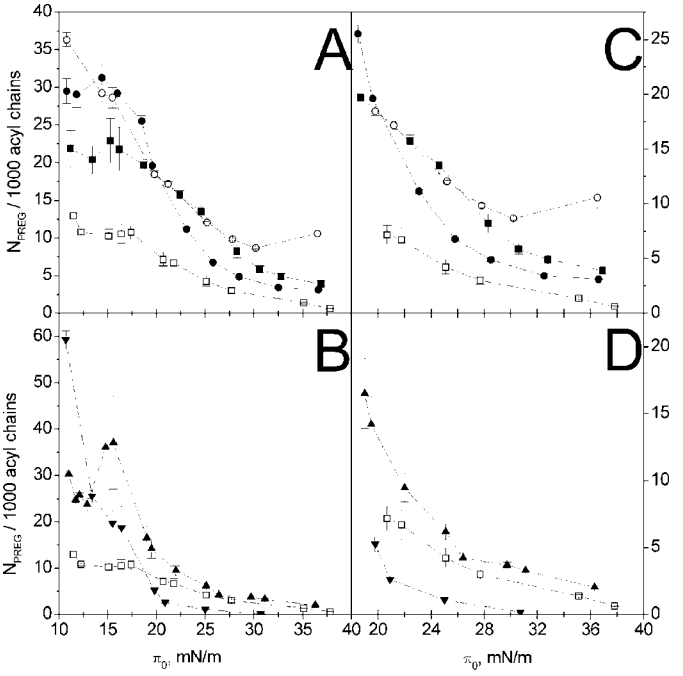


Fig. 6. Estimated lower limits for the number of pregnanolone molecules penetrating into lipid monolayers. [Pregnanolone] = 2 μM. Calculations were performed as described under *Materials and Methods*. A, the effect of increasing head-group spacing by POPC and cholesterol is shown. POPC (○), DPPC (□), DPPC/cholesterol 80:20 (■), and DPPC/cholesterol 60:40 (●) (mol/mol). B, the effect of dipole potential modulators is illustrated. The series are DPPC/phloretin 80:20 (▼), DPPC (□), and DPPC/6-ketocholestanol 80:20 (▲). The high π data region of A and B, which is devoid of phase transitions and more closely resembles bilayer state, is shown in C and D, respectively.

≈10 mN/m (Möhwald, 1995). No effects of phase transitions were observed in $\Delta\pi$ versus π_0 plot; there were no discontinuities in the slope. Yet, to exclude possible involvement of phase changes on the monolayer penetration of pregnanolone, we also studied POPC films, yielding at room temperature continuous liquid-expanded compression isotherms with no phase transitions (Smaby et al., 1994). Compared with DPPC, higher $\Delta\pi$ values were observed for POPC at all π_0 values studied (Fig. 5).

Discussion

Pregnanolone binds to lipid membranes, as revealed by the current monolayer experiments and differential scanning calorimetry measurements (data not shown). The membrane localization of pregnanolone was studied using probes for the hydrocarbon and interfacial regions, DPH and Prodan, respectively. Pregnanolone had an insignificant effect on DPH anisotropy (Fig. 3). Because this parameter assesses the acyl chain order of lipid membranes, our results demonstrate pregnanolone to have little effect on the dynamics of the membrane hydrocarbon region. In contrast, pregnanolone produced pronounced changes in Prodan fluorescence, indicating this steroid to partition into the interfacial region of the bilayer. Although effects of pregnanolone on the membrane association of Prodan may be involved, the GP data could also be partly explained by a tendency of pregnanolone to displace water from the lipid head groups in a manner similar to that of anesthetics (Tsai et al., 1990) and those alcohols which induce anesthesia.

The indirect assessment of the membrane dipole potential showed pregnanolone to decrease Ψ in a concentration-dependent manner, as shown previously for other general anesthetics (Cafiso, 1998). Because of the large scattering of the concentrations of pregnanolone used in vitro and in vivo (0.1–100 μM), as well as the difficulty of obtaining relevant lipid concentrations for in vivo measurements, the clinical relevance of this finding is difficult to estimate. Yet our data show pregnanolone to localize into the lipid-water interface and to induce changes in dipole potential. Penetration of pregnanolone into lipid monolayers did depend on Ψ. More specifically, the association of pregnanolone to different lipid films decreased in the order of POPC ≈ DPPC/cholesterol (80:20) > DPPC/cholesterol (60:40) > DPPC/6-ketocholestanol (80:20) > DPPC > DPPC/phloretin (80:20). The values for Ψ decrease in the order of DPPC/6-ketocholestanol (80:20) > DPPC/cholesterol (60:40) > DPPC/cholesterol (80:20) > DPPC > POPC > DPPC/phloretin (80:20). Changes in membrane association caused by modification of the monolayer composition with 6-ketocholestanol and phloretin suggest that Ψ is an important determinant for the membrane partitioning of pregnanolone. This effect of Ψ on pregnanolone partitioning can be easily rationalized, as follows. With increasing dipole potential and increasing repulsion between the partial charges of the dipoles contributing to the dipole potential, the dipole potential-decreasing effect of the oppositely oriented pregnanolone dipole becomes energetically more favorable, and the partitioning of pregnanolone into the membrane increases. In other words, the membrane association of pregnanolone is affected by dipole-dipole interactions. For pregnanolone, this effect could be more significant than for many other substances, because pregnanolone seems to reside mostly in the interface and not in the hydrophobic core, as evidenced by the lack of effects on DPH anisotropy.

The correlation between Ψ and ΔG for the binding of hydrophobic cations and anions to bilayer has been assessed (Franklin and Cafiso, 1993). In brief, the more negative interfacial dipole potential caused by phloretin (X = 0.10) produced a 2- to 3-fold decrease in the binding of hydrophobic anions. Although the ions used by Franklin and Cafiso are hydrophobic compared with metal ions, their free-energy minima should be found in the interface, because the transfer of free charges into the very apolar interior ($\epsilon_r \approx 2$) of bilayers would increase ΔG because of Born energy. Because dipole-dipole interactions are weaker than ion-dipole interactions, the effect of dipole potential on the binding of dipoles could be expected to be smaller. However, the changes in the binding of pregnanolone seem to be of the same order of magnitude. The high degree of anisotropy of drug dipoles as well as lipid and water dipoles in the membrane may provide the mechanistic basis for this. More specifically, when the orientation of dipoles is more or less fixed and the distances between the dipoles are not long compared with the separation of partial charges within a dipole, then the partial charges of different dipoles interact strongly and can in effect be considered as separate charges. For a weakly amphiphilic compound such as pregnanolone, ΔG for binding receives favorable entropy contribution because hydrophobic parts of the molecule are removed from water. An unfavorable contribution comes from polar groups, because enthalpy of dipolar interactions decreases when freely oriented water dipoles from the surroundings of the polar groups are replaced by membrane dipoles. A number of other contributions are likely to be involved, but as a first approximation, we can concentrate on the above. If the dipole-dipole interactions between the drug molecule and membrane change, they can be optimized by reorientations and/or changes in the position of pregnanolone as well as lipids in the bilayer. Such alterations are likely to affect also the surroundings of buried apolar groups of the drug molecule and therefore the entropic and hydrophobic contributions to ΔG. Therefore, changes in membrane dipole potential changes cannot be completely compensated, and ΔG for a drug in membrane and in water is altered (the partition coefficient changes).

Our results further demonstrate that the lipid head-group spacing is an important additional determinant for the membrane partitioning of pregnanolone. This is evident from the finding that pregnanolone partitions more avidly to cholesterol-containing monolayers for which the phospholipid head group spacing is higher, but Ψ is lower than for 6-ketocholestanol–containing films. In addition, despite practically equal effective dipole moments of DPPC and POPC (469 and 468 mD), and therefore higher Ψ values for DPPC caused by weaker packing of POPC at equal surface pressures (Smaby and Brockman, 1990; Smaby et al., 1994), pregnanolone seems to favor partitioning into POPC monolayers. In keeping with the weaker lateral packing (higher area per molecule) for POPC, one would expect larger head-group spacing and free volume in the head-group region, and therefore more efficient binding for pregnanolone, as observed. This suggests the head-group spacing (free volume) of the monolayer to be an important determinant of pregnanolone membrane association. In POPC membranes, the lateral spacing between

hydrophilic head groups is higher because of the acyl chain *cis*-double bond, which leads to increased hydration (Jendrasiak and Hasty, 1974). In addition, the presence of additives such as cholesterol increases the distance between adjacent head groups (Jendrasiak and Hasty, 1974). Because pregnanolone partitions into the interfacial region, these non–Ψ-dependent effects can be explained by the larger head-group spacing in lipid membranes containing cholesterol or unsaturated lipids (such as POPC). Our results are in perfect agreement with Monte Carlo simulations on DMPC/cholesterol bilayers. More specifically, cholesterol was found to increase the number of spherical cavities in the polar region of bilayers and thus to lower the solvation free energy for small molecules in this region (Jedlovszky and Mezei, 2003). In effect, pregnanolone could be seen as a weak amphiphile that partitions into the regions of the lipid-water interface insufficiently covered by lipid polar head groups. In conclusion, our current results suggest that membrane partitioning of pregnanolone is influenced by multiple factors, namely lipid lateral-packing density, phospholipid head-group spacing, and membrane dipole potential.

Despite the lack of the knowledge about the exact mechanism of general anesthesia, the anesthetic-lipid interactions are important for several reasons. First, to access target(s) in the central nervous system, a compound has to cross several cellular membranes, including the blood-brain barrier. In the process of cell-membrane permeation, the drug-lipid interactions are crucial. In addition, the site of action for anesthetics is the plasma membrane, in which the primary target is either the lipid phase or integral membrane proteins. Especially in the former case, the drug-lipid interactions are of importance. Yet, because these interactions determine the partitioning of anesthetic to the membrane, they are of significance also when the target is an integral membrane protein. Furthermore, the existence of different lateral membrane domains having distinct lipid and protein compositions suggests that the differences in membrane partitioning might also affect the availability of a substance to proteins in these membrane domains. Only small numbers of most individual integral membrane protein species are present in the cell membrane, whereas the numbers of different lipids are large. From a speculative standpoint, factors such as those presented here might enable different membrane lipid domains to serve as attractors gathering certain membrane-soluble ligands or substrates and enriching them into (or depleting from) the vicinity of the target proteins, thus affecting the effective ligand concentration within the target protein containing membrane domains. Therefore, whether anesthesia is mediated by specific binding to receptors or by nonspecific or indirect effects on membrane proteins, the availability of anesthetic at the lipid-water or lipid-water-protein interface could be modulated by properties of the domains. As such, the integral membrane proteins act as perturbants for lipids and are likely also by themselves to create more space into the region having the permittivity of the lipid-water interface. It has been suggested (Cafiso, 1998; Ueda and Yoshida, 1999) that the main membrane effects of anesthetics relevant to anesthesia would not be related to the decrease in acyl chain order and increase in lateral diffusion, as was assumed previously. To this end, the importance of the magnitude and the orientation of interfacial anesthetic dipoles could, hypothetically, also explain why some pregnanolone stereoisomers possess slightly different anesthetic activities (Covey et al., 2000).

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