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Lack of enantiomeric specificity in the effects of anesthetic steroids on lipid bilayers

Juha-Matti Alakoskela [a,*], Douglas F. Covey [b], Paavo K.J. Kinnunen [a,c]

[a] *Helsinki Biophysics and Biomembrane Group, Institute of Biomedicine/Biochemistry, P.O. Box 63, 00014 University of Helsinki, Finland*
[b] *Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, USA*
[c] *MEMPHYS—Center for Biomembrane Physics, Odense, Denmark*

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Abstract

The most important target protein for many anesthetics, including volatile and steroid anesthetics, appears to be the type A γ-amino butyric acid receptor ($GABA_AR$), yet direct binding remains to be demonstrated. Hypotheses of lipid-mediated anesthesia suggest that lipid bilayer properties are changed by anesthetics and that this in turn affects the functions of proteins. While other data could equally well support direct or lipid-mediated action, enantiomeric specificity displayed by some anesthetics is not reflected in their interactions with lipids. In the present study, we studied the effects of two pairs of anesthetic steroid enantiomers on bilayers of several compositions, measuring potentially relevant physical properties. For one of the pairs, allopregnanolone and *ent*-allopregnanolone, the natural enantiomer is 300% more efficacious as an anesthetic, while for the other, pregnanolone and *ent*-pregnanolone, there is little difference in anesthetic potency. For each enantiomer pair, we could find no differences. This strongly favors the view that the effects of these anesthetics on lipid bilayers are not relevant for the main features of anesthesia. These steroids also provide tools to distinguish in general the direct binding of steroids to proteins from lipid-mediated effects.

Keywords: Pregnanolone; Allopregnanolone; Dipole potential; Lateral pressure profile; Lipid phase transition

1. Introduction

In a related study we demonstrated a pronounced enantiospecificity in the effects of two pairs of enantiomeric anesthetic steroids on $GABA_C\rho_1$ receptors, suggesting that the effect is mediated by direct interaction with stereospecific sites on proteins [1]. However, like proteins, the membrane lipids are optically active, and the lack of enantiospecificity with lipids must be demonstrated to confirm the conclusion of direct interaction with proteins. Because an earlier report of receptor mutants found that the different effects of 5β- and 5α-neurosteroids (such as pregnanolones and allopregnanolones, respectively) were incompatible with direct binding to the two amino acids that are critical for the effects of steroids and that are located in the transmembrane segments 2 and 3, the changes caused by amino acid substitutions led Morris and Amin to conclude that these critical amino acids act as sensors of local polarity and pressure profile [2].

Issues concerning the enantiospecificity of lipid interactions are also connected to the questions about the mechanisms of general anesthetics. Different groups of anesthetics affect a different spectrum of proteins. Volatile anesthetics of the group, including halothane, isoflurane, and sevoflurane, have perhaps the widest range of effects at clinically relevant concentrations, as they decrease transmitter release, increase activation of $GABA_A$ receptors (with no significant effects on NMDA or AMPA receptors), affect signal integration by activating K^+ channels and inactivating Na^+ channels, and decrease axonal conductance [3,4]. One of the suggested primary targets of anesthetics is the type A γ-amino butyric acid (GABA) receptor, a chloride channel gated by its ligand GABA. Yet, attempts to demonstrate the actual binding by photolabelling a specific site on $GABA_AR$ with azipregnanolone or with other anesthetic derivatives have not yielded positive results [5–7], though various residues can be labeled by anesthetics of many

* Corresponding author. Tel.: +358 9 19125426; fax: +358 9 191 25444.
E-mail address: Juha-Matti.Alakoskela@helsinki.fi (J.-M. Alakoskela).

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groups in ligand-gated ion channels that are available in larger quantities [8–10]. At present the role of the labeled amino acids in anesthesia remains uncertain. Nevertheless, many, if not most, anesthetics do increase the chloride currents by activating $GABA_A$ receptors either directly or indirectly, and the magnitude of the effect on $GABA_A$ receptors correlates well with anesthetic potency [11], and with the enantiospecificities of etomidate [12], steroid anesthetics [13], and barbiturates [14]. The level of enantiospecific effects between groups varies. For the volatile anesthetic, isoflurane, the *R*-enantiomer is typically reported to have 1.4–1.6 fold greater potency [15–17] (with one exception [18]) in both animals and on some ion channels in vitro [15,19,20]. The *R*-enantiomer of halothane has been reported to have, depending on the strain of *C. elegans* used, 1.0–3.0 fold greater potency compared to *S*-halothane [21]. Differences between enantiomers are larger for other groups of anesthetics, up to 10 fold [6,13].

Surprisingly, though numerous exceptions exist, one unifying property of many anesthetics is that their potency is proportional to the olive oil/water partition coefficient. This is the so-called Meyer–Overton rule [3,22,23]. This simple correlation is enticing, and some still suggest that actions on these proteins are mediated by lipid membranes [24–27] or by a simple physical mechanism such as a change in the hydration of lipids and proteins [22]. The lipid environments of a protein affect its functions and its mode of action [28], and, hypothetically, anesthetics could modulate these environments. The suggested mechanisms of lipid-mediated action include the effects of anesthetics on dipole potential and spontaneous curvature [24], or on the lateral pressure profile [25–27,29–31]. Indeed, while prevailing anesthetic hypothesis favors direct binding to proteins, many proponents of lipid mediated action exist.

While both the direct-binding and the lipid-mediated hypotheses of anesthetic action can explain the specific actions on specific proteins, the cut-off sizes for the anesthetic potency, and other deviations from the Meyer–Overton rule [29,30,32–37]; the sum of all the evidence points towards direct actions on proteins. In particular, no optically active anesthetic has been shown to have enantiospecific interactions with membrane lipids [12,38]. A *caveat* is that the effects on many properties suggested to be important for the hypotheses of lipid-mediated anesthesia were not studied and only a few phospholipid compositions were used. The lack of enantiospecific interactions is by no means obvious *a priori*, as phospholipids and cholesterol are chiral molecules known to display enantiospecific interactions [39]. The enantiospecificity of the interactions of steroid anesthetics with lipids has not been studied, while there exists a body of literature showing the correlation of the anesthetic potency of steroids with the effects on hydration, the effects on lipid bilayers, and the structure and function of model proteins and peptides [40–47]. While the simple Meyer–Overton rule describing the correlation of anesthetic potency with solubility in bulk solvent does not extend very well to complex, large compounds like steroids, the spirit of the rule is obeyed by steroids, as the effects on bilayers have been reported to correlate well with the anesthetic potency [43], and the effects

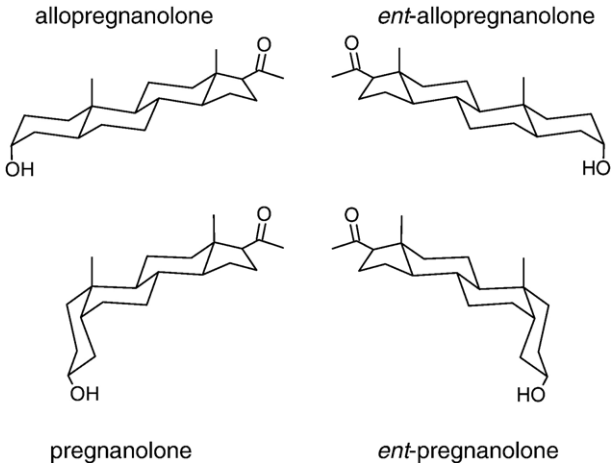

Fig. 1. Structures of steroids. *Ent*-allopregnanolone and *ent*-pregnanolone are enantiomers, i.e., mirror image isomers, of allopregnanolone and pregnanolone, respectively.

of the anesthetic steroids and the inhaled/volatile/gaseous anesthetics on model proteins and lipid bilayers are qualitatively similar [40–47]. It should be kept in mind, however, that these are clearly separate groups of anesthetics (see Discussion), and any comparison should be made with extreme caution.

In this study, we examined the interactions of two pairs of enantiomers with lipid bilayers (Fig. 1). One of the pairs, allopregnanolone and *ent*-allopregnanolone, shows a high degree of enantioselectivity in anesthetic effects, while the other pair, pregnanolone and *ent*-pregnanolone, displays minimal enantioselectivity. Additionally, the potency of the pregnanolone enantiomer pair is very close to that of natural allopregnanolone [13]. If any of the membrane properties are significant for anesthesia, we would expect a stronger effect for the pregnanolones (natural and *ent*) and natural allopregnanolone, and a considerably weaker effect for *ent*-allopregnanolone. We tested several lipid bilayer compositions to cover major lipid classes in plasma membranes, and one readily available biomembrane to check the effects in a complex mixture. We found no enantiospecificity with respect to any bilayer property measured or any bilayer composition tried. Accordingly, lipid-mediated actions by these steroids are highly unlikely to explain the enantiospecificity of steroid-induced anesthesia, enantioselective effects on $GABA_AR$ [13], or the enantiospecific effects on $GABA_C\rho_1R$ [1].

2. Materials and methods

2.1. Materials

Phospholipids were from Avanti Polar Lipids (Alabaster, AL, USA). (3α,5β)-3-Hydroxypregnan-20-one (pregnanolone) and (3α,5α)-3-hydroxypregnan-20-one (allopregnanolone) were obtained from Steraloids (Wilton, NH, USA). *Ent*-pregnanolone and *ent*-allopregnanolone were synthesized as described previously [48,49]. 6-Lauryl-2-dimethylaminonaphthalene (Laurdan) and 4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl)-pyridinium (di-8-ANEPPS) were from Molecular Probes (Eugene, OR, USA), and 1,2-bis[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine (bisPDPC) was

from K and V Bioware (Espoo, Finland). Other reagents were from Sigma or Merck. Deionized water was Millipore filtered (Millipore, Bedford, MA, USA). Lipids appeared as a single spot in TLC. The lipid stock solutions were made in chloroform, and the concentrations of non-fluorescent and fluorescent lipids were determined gravimetrically using a Cahn 2000 electrobalance (Cahn Instruments, Inc., Cerritos, CA, USA) and photometrically using the molar absorptivities provided by Molecular Probes, respectively.

2.2. Preparation of liposomes

Lipids (and dyes) were mixed in chloroform to yield the desired molar ratios. Phospholipid:probe ratio was 100:1 for samples with Laurdan and di-8-ANEPPS samples, and 1000:1 for samples with bisPDPC. The mixtures were dried under a stream of nitrogen, then kept under reduced pressure for at least 2 h. The samples were hydrated in a buffer (5 mM HEPES, 0.1 mM EDTA, pH 7.4) for 60 min at approximately 60 °C to yield multilamellar liposomes (MLVs). In order to obtain large unilamellar vesicles (LUVs) the MLV dispersions were subsequently extruded through a polycarbonate filter (pore size 0.1 μm, Millipore, Bedford, MA, USA) using Liposofast-Pneumatic (Avestin, Ottawa, Canada).

2.3. Preparation of erythrocyte ghost samples

Blood samples were collected from the corresponding author. Red blood cells were isolated by centrifugation, and repeatedly washed using 5 mM Na_2HPO_4 buffer, pH 8.0, containing 150 mM NaCl, until the supernatant was clear. A 1× volume of RBCs was added to a 20× volume of hypotonic buffer (5 mM Na_2HPO_4, pH 8.0) and incubated for 10 min on ice to burst the cells. RBC ghosts were pelleted by centrifuging for 3 min at 20000×*g*, and washed by resuspending and repelleting until the remaining ghosts were pearly gray.

To evaluate the lipid content of the ghost sample, 1× volume of ghosts was mixed with 4× volumes of 2% (w/v) NaCl, followed by addition of 5× volume of chloroform and 10× volume of methanol, and further rigorous mixing, and centrifugation for 5 min with a small table centrifuge at maximum rpm. Liquid was then removed to a new tube, 5× volume of both chloroform and water was added, and centrifugation was repeated. The lipid mass in the chloroform phase was determined gravimetrically, and the lipid concentration was estimated using an average molar weight of 700 g/mol. The ghosts were suspended in buffer (5 mM HEPES, 0.1 mM EDTA, pH 7.4) to give the desired lipid concentration. Dyes in DMSO were added to give lipid/dye molar ratios identical to those in LUVs, and 1% (v/v) DMSO. Steroid solution in DMSO was then added to yield the desired steroid concentration, and 2% (v/v) total concentration of DMSO. The samples were then incubated overnight on ice before the measurements.

2.4. Fluorescence spectroscopy

A series of steroid solutions in DMSO was prepared to give 1% DMSO and the desired steroid concentrations in the samples. These steroid solutions were added onto LUVs or ghosts and incubated overnight on ice before measurements. Measurements were made in triplicate, except in duplicate for ghosts:di-8-ANEPPS samples. Unless otherwise mentioned, all error bars represent the sample standard deviation of three independent samples. Because of the small number of samples the sample standard deviation may not reflect the true population standard deviation.

Fluorescence was measured with a Varian Cary Eclipse fluorometer equipped with a temperature-controlled four-cell cuvette holder and an immersible temperature sensor. In each measurement three cuvettes contained samples and the fourth cuvette contained an approximately equal volume of water with a temperature probe immersed in water. With each membrane matrix, for each fluorophore and for each steroid anesthetic concentration the measurements were done at temperatures equilibrated at 10, 20, 30, 40, 50, and 60 °C.

For different ratiometric dyes di-8ANEPPS, Laurdan and bisPDPC, excitation (di-8-ANEPPS, λ_{em}=670 nm) or emission (Laurdan, λ_{ex}=350 nm, and bisPDPC, λ_{ex}=344 nm) spectra were collected. To ensure optimal signal-to-noise ratios for the intensity ratios the intensity at each of the ratio wavelengths was integrated for a total of 15 s in interspersed intervals of 1.0 s. From these values, the di-8-ANEPPS intensity ratio R is calculated according to

$$R = I_{440}/I_{530}. \quad (1)$$

The value of R has been shown to correlate well with dipole potential with little interference from other properties such as surface charge [50–52]. In order to evaluate the corresponding dipole potential values, a calibration series using the same settings was measured with the known dipole potential modulators phloretin and 6-ketocholestanol, as described previously [53]. Despite the error sources [54] of the reference method [55] the magnitude of the changes in dipole potential should be fairly accurate. Some error is introduced by fitting the curve with a single exponential function, as this fitting method somewhat overestimates dipole potentials between 150 and 250 mV and underestimates dipole potentials between 250 and 350 mV, but provides a reasonable fit for the whole range of values. It can be seen from the examples of calibration curves in Fig. 2 that the magnitude of the change in R by both 6-KC (Ψ>280 mV) and PHLOR (Ψ<280 mV) decreases at higher temperatures, likely reflecting either temperature-dependent changes in probe fluorescence or increased water solubility of 6-KC and PHLOR. To illustrate the effects of steroids, the differences in Ψ between the pure (no steroid) and the steroid-containing bilayers were calculated separately for each series and only then averaged.

The GP value for Laurdan is calculated as

$$GP = (I_b - I_r)/(I_b + I_r), \quad (2)$$

where I_b indicates the intensity at the blue-edge wavelength (440 nm) and I_r the intensity at the red-edge wavelength (490 nm). This Laurdan excitation GP value is mainly sensitive to probe ground state hydration [56], which in turn is to a large extent dependent on the water penetration to the level of ester carbonyl groups where Laurdan resides. For a series of similar phospholipids (such as anionic, glycolipids or zwitterionic) this leads to a linear relationship between the lipid interbackbone distance and Laurdan GP value [57], which has been utilized in measurements of lipid molecular area [58].

The probe bisPDPC contains two pyrene moieties attached to decanoyl chains. The radiative relaxation of pyrene monomer gives fluorescence at 398 nm ($\rightarrow I_m$). If, however, an excited pyrene collides with a ground state pyrene, an excited dimer or excimer is formed, and the emission of this excimer is centered at 480 nm. For bisPDPC most of the collisions occur intramolecularly, and accordingly, the ratio I_e/I_m mostly reflects acyl chain

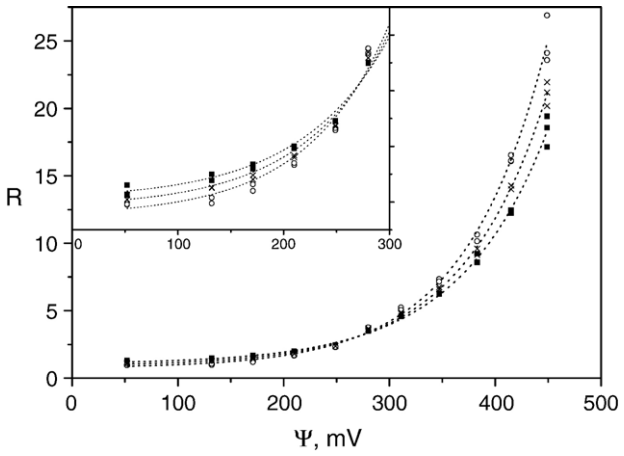

Fig. 2. Examples of calibration curves for di-8-ANEPPS based Ψ evaluation. Measurements at 20 °C (○), 30 °C (×) and 40 °C (■). Inset shows low dipole potential region. Matrix lipid was POPC, mixed with 6-KC or PHLOR. Pure POPC has Ψ=280 mV. Dotted lines represent fits by single exponential. Please notice that for the sake of simplicity x-values were taken to be identical at different temperatures.

mobility and the average volume occupied by the acyl chains (or free volume) [59,60]. For unknown reasons, these parameters correlate to some extent with local lateral pressure, as some success in evaluating the shape of the lateral pressure profile was achieved with bis-(pyrene-acyl)-PCs of different acyl chain lengths [61]. Together with Laurdan GP, bisPDPC I_e/I_m thus could respond to at least large changes in the lateral pressure profile, though the profile itself, of course, cannot be estimated based on the results.

2.5. DSC

The effect of steroids on phase transitions was studied by a microcalorimeter (VP-DSC, Microcal Inc.). 1.0 mM phospholipid was used as multilamellar vesicles. Steroid was added in chloroform and mixed with phospholipid in chloroform, then dried under a nitrogen stream, and MLVs were prepared as described above. Each sample was used right after the hydration without an incubation.

3. Results

There was no significant difference between the steroid enantiomers in any of the measurements at any temperature, whereas there are both quantitative and qualitative differences between the 5β-reduced pregnanolones (pregnanolone and *ent*-pregnanolone) and the 5α-reduced pregnanolones (allopregnanolone and *ent*-allopregnanolone). Because of the large number of studies done only a representative cross-section of data is presented here.

The reader should note that the partitioning of steroids into vesicles was not assessed. If any difference would exist between enantiomers, it would not matter whether it would be present due to a different partitioning or due to the different effects at equal partitioning. As no difference in any property is found, the most likely conclusion is that the partitioning is equal between enantiomers.

3.1. Dipole potential by di-8-ANEPPS

Pregnanolones and allopregnanolones had almost equal effects on dipole potential in most membranes made of synthetic lipids (Fig. 3). As the calculated ratio showed some between series variation, the following normalization was done to obtain a better estimate of the effects of steroids. First, the use of two zero samples in each series gave within-series-error, which was combined with the error in the decrease due to steroids. In Fig. 3, data are presented for change at 30 °C anesthetic:phospholipid ratios of 1:100 and 5:100, which are the lowest used. The decrease in dipole potential for the cholesterol-containing bilayers appears slightly larger, possibly because cholesterol increases the partitioning of steroids [53]. However, this effect is stronger for the allopregnanolones than for the pregnanolones, and the allopregnanolones decrease the dipole potential of the cholesterol-containing bilayers more efficiently. It can also be seen that the effects on erythrocyte ghosts are smaller than those on most synthetic bilayers. In ghosts there is a large difference between pregnanolones and allopregnanolones, the latter decreasing the dipole potential of erythrocyte ghosts approximately twice as effectively. Lipids or proteins missing from synthetic membranes, such as glycolipids and phosphoinositols, could also favor the increased binding of allopregnanolone, or might affect the relative orientations of the steroids.

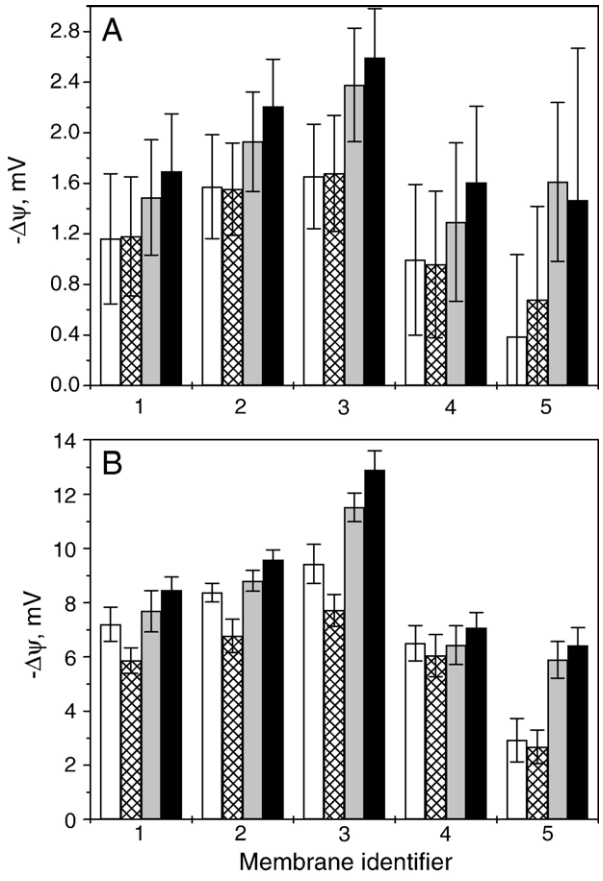

Fig. 3. Effect of steroids on Ψ in different membranes. Membrane identifiers 1, 2, 3, 4 and 5 represent POPC, POPC:CHOL=70:30, POPC:Brain-SM:CHOL=35:35:30, POPC:POPE:POPS=30:30:40 and RBC ghosts, respectively, all with c=160 μM. White, hatched, light gray and dark gray columns represent PREG, *ent*-PREG, ALLOPREG and *ent*-ALLOPREG, respectively. Temperature=30 °C. In panels A and B the effects of 1.6 and 8.0 μM steroid are shown, respectively.

An additional feature appears to be that at the highest steroid concentrations used (32 μM, drug/lipid=1/5) the effect of the pregnanolones on dipole potential remains approximately the same at different temperatures while the effect of allopregnanolones increases slightly. As this finding is observed in all the experiments, it is likely true. For example, pregnanolones (32 μM) decrease Ψ by 24±3 mV at 10 °C and by 26±2 mV at 60 °C in a POPC matrix,, whereas allopregnanolones decrease Ψ by 13.6±0.8 mV at 10 °C and by 27±3 mV at 60 °C (Fig. 4). It should be noted that the calibration assumes a nonphysical, temperature-independent dipole potential for the calibration compositions. Accordingly, any temperature-dependent changes of the calibration samples are super-imposed on the temperature-dependent changes of the steroid-containing samples. However, this does not affect the differences in the temperature response between the different steroids. Possible explanations are a different temperature dependence of lipid partitioning for the pregnanolones and the allopregnanolones, or different temperature-

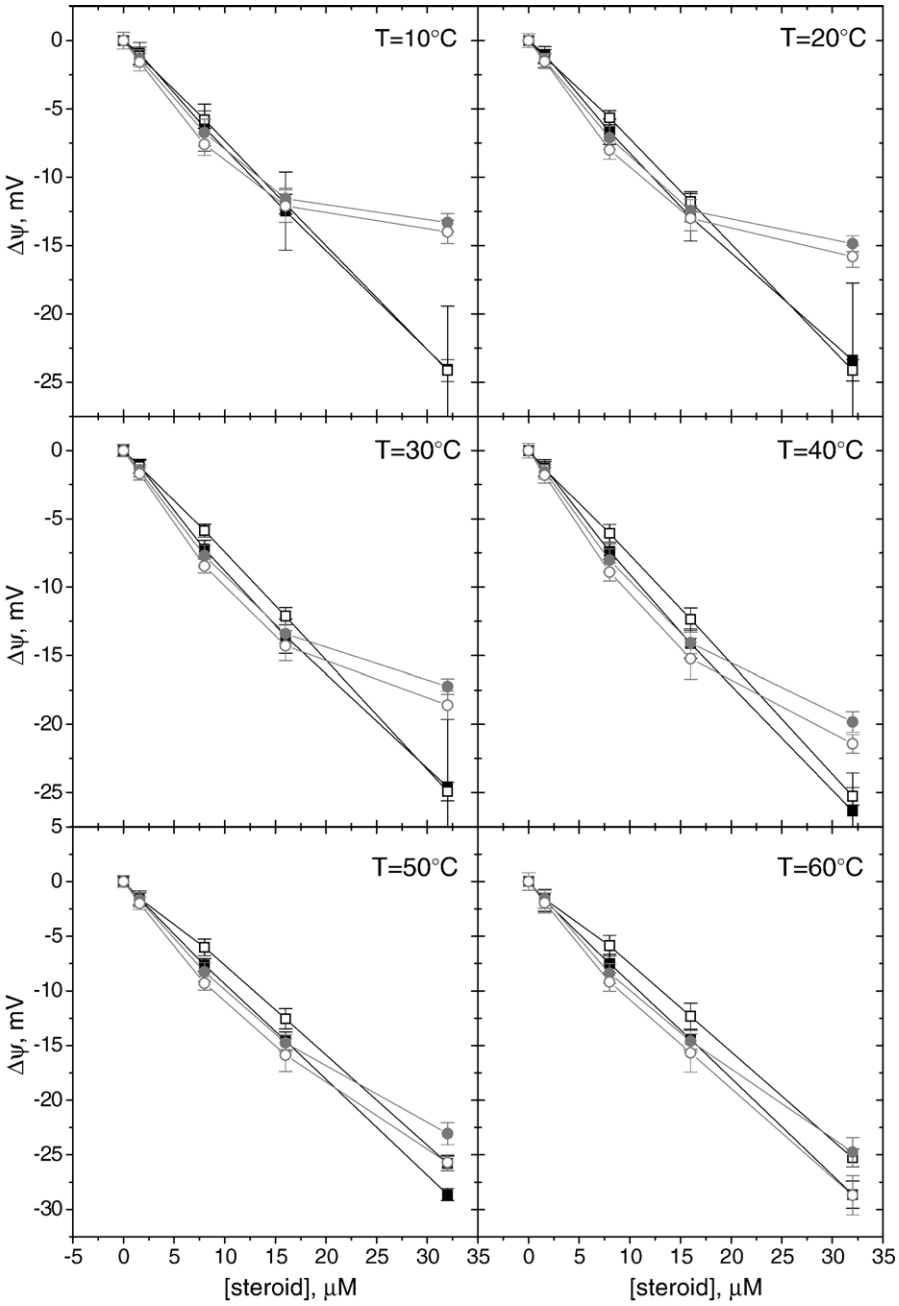

Fig. 4. Effects of anesthetic steroids on Ψ of POPC bilayers at different temperatures. Black filled squares and open squares represent pregnanolone and *ent*-pregnanolone, respectively, while gray filled and open circles represent allopregnanolone and *ent*-allopregnanolone, respectively.

dependent effects on acyl-chain orientation or order for the steroid-containing membranes.

3.2. Interfacial hydration/free volume by Laurdan

An increase in Laurdan GP value typically implies a decreased interaction of the fluorescent moiety with water [56]. This results from either a decrease of the interlipid distance at the depth of a lipid carbonyl group where the fluorescent moiety of Laurdan resides [57], or from the dehydration of membranes, i.e. displacement of water from this region by some solute that has weaker interactions with Laurdan. The increase in Laurdan GP value when going from fluid phase to gel phase corresponds very well with the decrease in area per lipid. In all membranes, allopregnanolones increased Laurdan GP values more than pregnanolones. Surprisingly, while in the POPC matrix pregnanolones also increased the GP value at all temperatures except 10 °C, in all the other synthetic membranes pregnanolones slightly decreased the GP value, this effect being strongest for a POPC:Brain-SM:CHOL=35:35:30 mixture. Most likely this result cannot be simply explained by the higher initial order of those membranes, as the same effect was seen for completely fluid POPC:POPE:POPS=30:30:40 membranes. Allopregnanolones

slightly increased GP in these systems, though their effect was not as strong as in the POPC matrix. Surprisingly, for the membranes of erythrocyte ghosts the Laurdan GP value exhibited behavior that was closest to that of POPC bilayers: both the pregnanolones and the allopregnanolones increased GP value, although for the pregnanolones the effect is very weak. The effects of steroids on Laurdan GP value at 30 °C in different bilayers are shown in Fig. 5. With the exception of POPC (left side panels of Fig. 6), the effects of allopregnanolones tended to increase compared to those of pregnanolones when temperature was increased, as an example some of the data for POPC:Brain-SM:CHOL=35:35:30 is shown (right side panels of Fig. 6).

3.3. Intramembrane free volume/chain mobility by bisPDPC

The effects of steroid anesthetics on the excimer to monomer ratio (I_e/I_m) of bisPDPC form the most complex set of results, as they show the largest differences in the diastereoselective (5α versus 5β) effects between the different bilayer compositions. In POPC membranes pregnanolones increase I_e/I_m considerably, while allopregnanolones cause

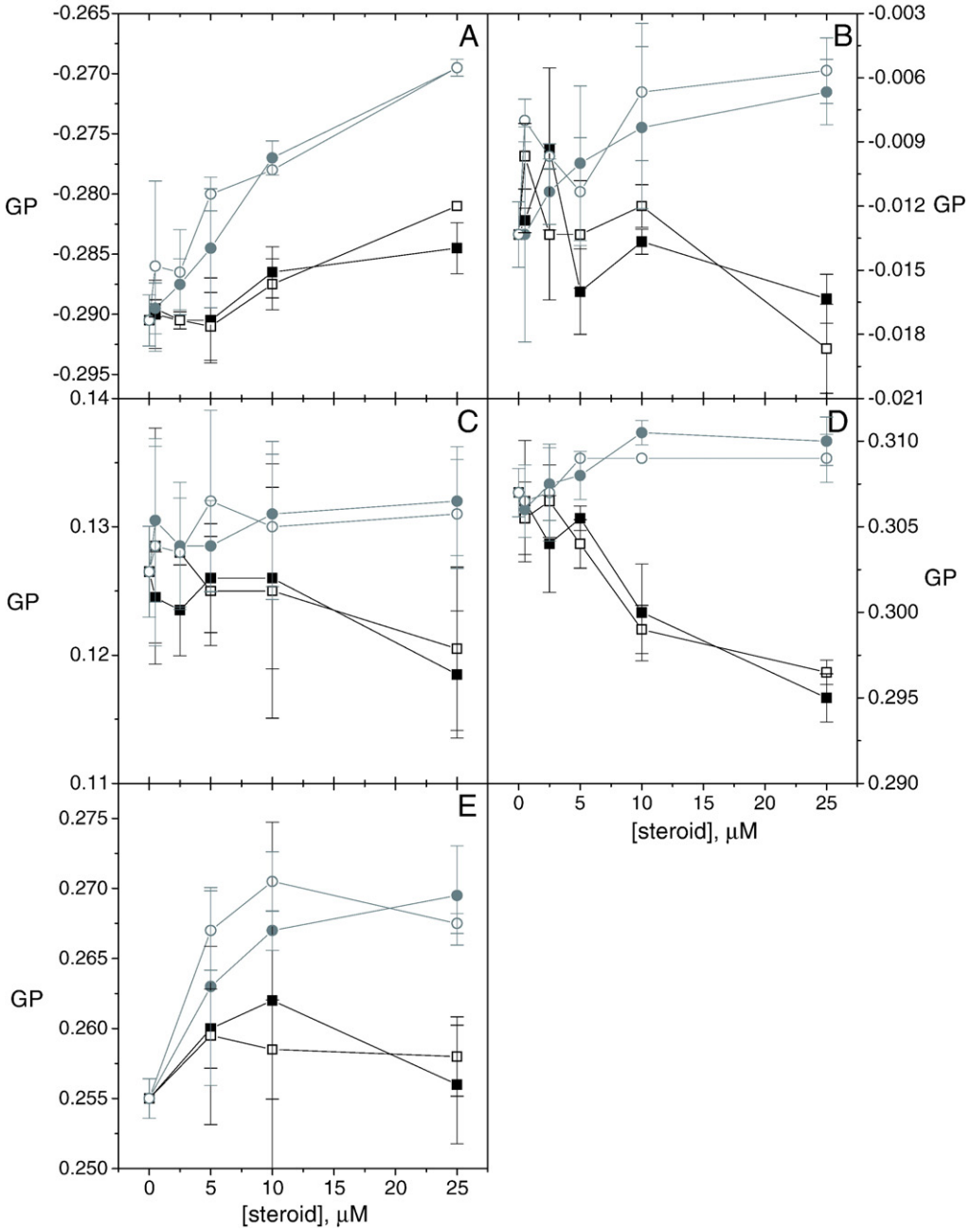

Fig. 5. Effects of steroids at 30 °C on Laurdan GP value in different bilayers. Black filled squares and open squares represent pregnanolone and *ent*-pregnanolone, respectively, while gray filled and open circles represent allopregnanolone and *ent*-allopregnanolone, respectively. Panels A, B, C, D and E show results for POPC, POPC:POPE:POPS=30:30:40, POPC:CHOL=70:30, POPC:Brain-SM:CHOL=35:35:30, and RBC ghosts. Phospholipid concentration is 50 μM.

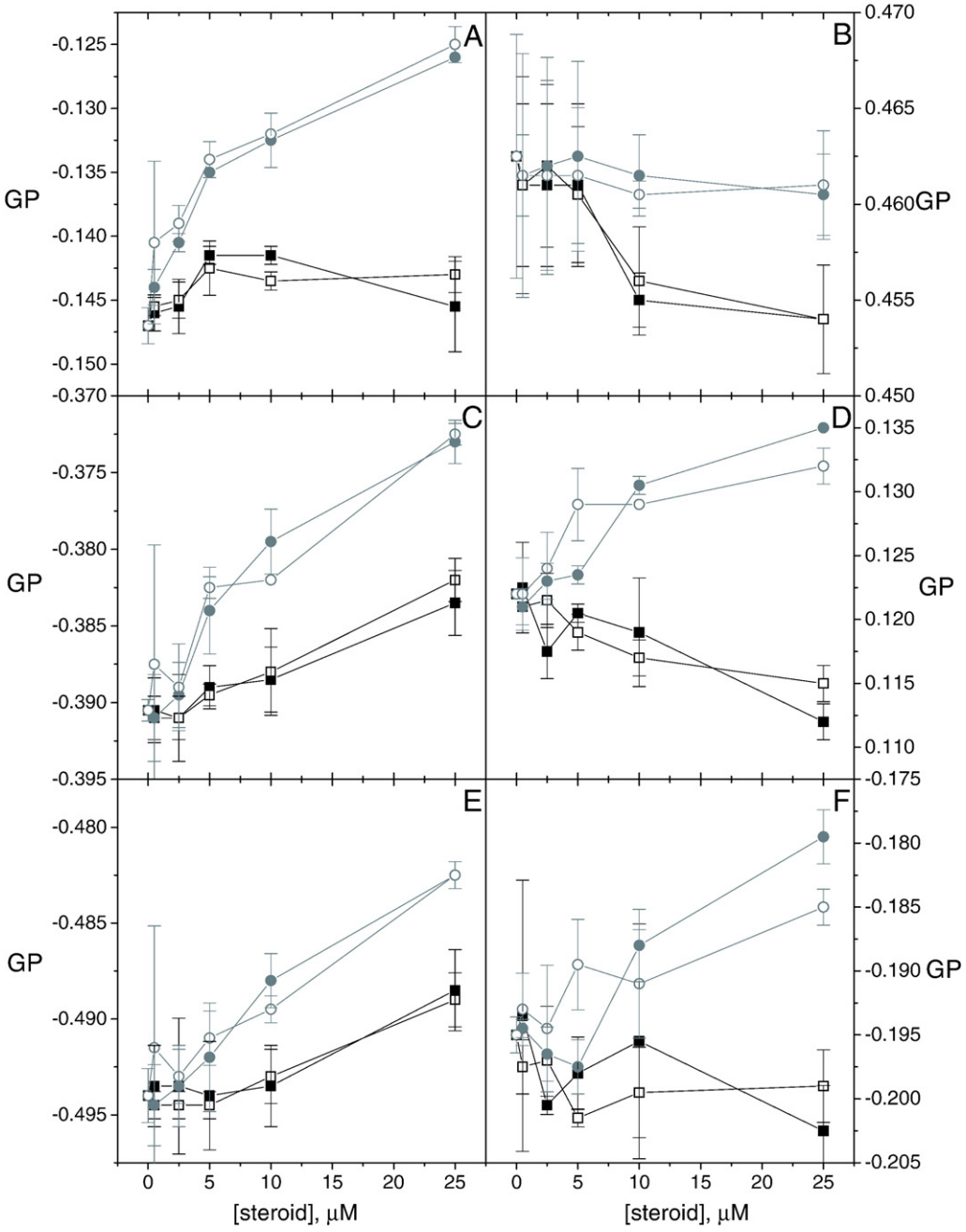

Fig. 6. Temperature dependent effects of steroid on Laurdan GP value portrayed for POPC (panels A, C, and E) and POPC:Brain-SM:CHOL=35:35:30 (panels B, D, and F) matrices. Panels A and B show the effects at 20 °C, panels C and D at 40 °C and panels E and F at 60 °C. Black filled squares and open squares represent pregnanolone and *ent*-pregnanolone, respectively, while gray filled and open circles represent allopregnanolone and *ent*-allopregnanolone, respectively.

only a small increase (Figs. 7 and 8, panel A). The effects on PC:CHOL=70:30 and PC:SM:CHOL=35:35:30 bilayers are more similar for the diastereoisomers: at low temperatures, neither pregnanolones or allopregnanolones have a significant effect (Fig. 7 panels C and D), while at higher temperatures the effects of pregnanolones are only slightly stronger than those of allopregnanolones, although especially in the case of POPC:CHOL the signal-to-noise ratio is very poor (Fig. 8 panels C and D). In POPC:POPE:POPS bilayers I_e/I_m was decreased slightly but consistently by allopregnanolones at low temperatures, though the decrease remains within the combined error bars, whereas pregnanolones appear to have a modest increasing effect or no effect (Figs. 7 and 8, panel B). In RBC ghost membranes pregnanolones have little effect on I_e/I_m, whereas allopregnanolones cause a decrease in I_e/I_m (Figs. 7 and 8, panel E), the effect being stronger when close to physiological temperatures (40 and 30 °C) (Fig. 8 panel E). Curiously, the change in I_e/I_m ratio for ghosts is an order of magnitude larger than changes in synthetic bilayers. Considering this, and that even the sign of the effect changes, it appears that in this respect the synthetic bilayers used do not provide a good model for a plasma membrane. Possibly

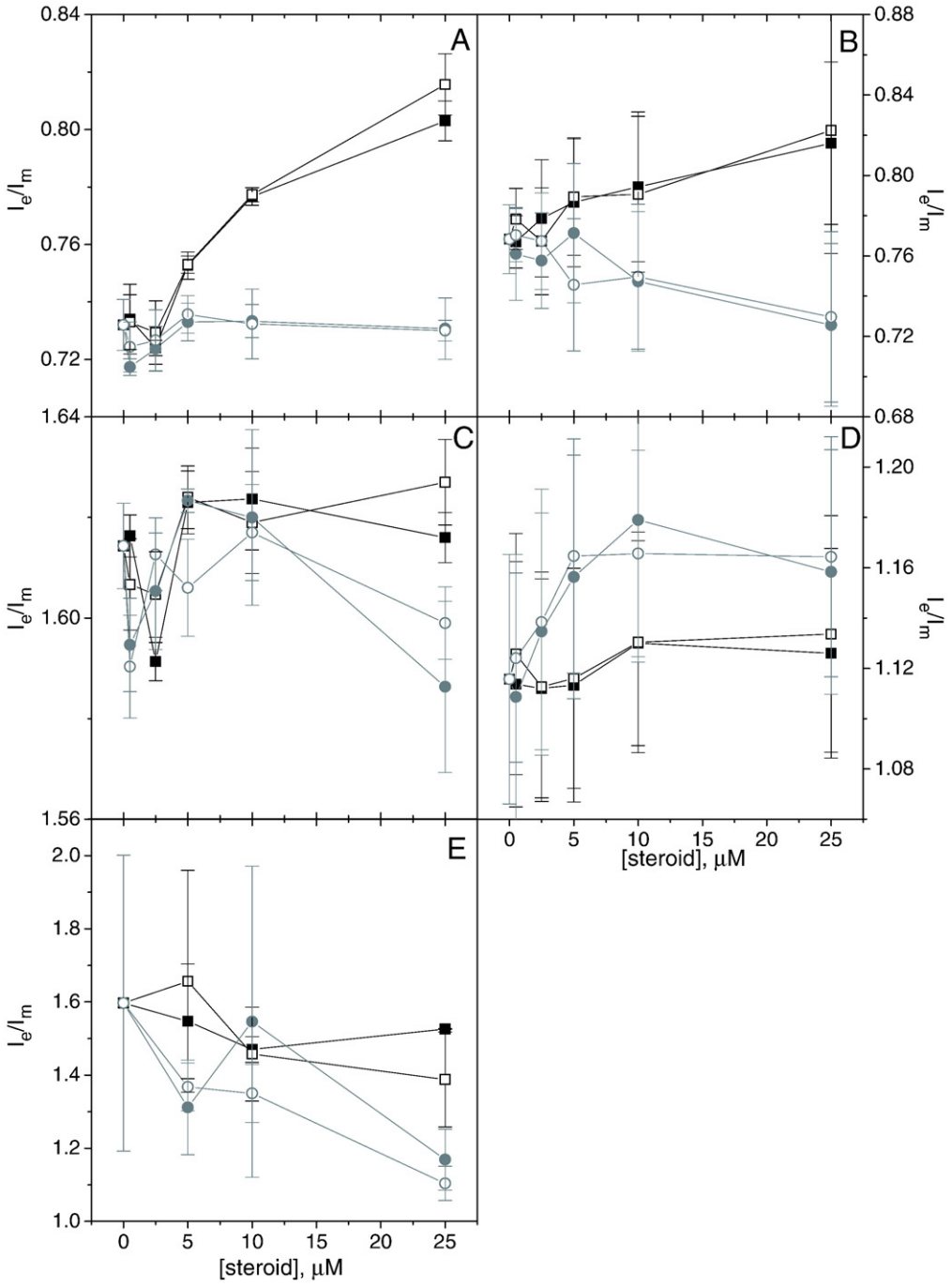

Fig. 7. The effects of steroids on I_e/I_m of bisPDPC in different bilayer matrices at 10 °C. Black filled squares and open squares represent pregnanolone and *ent*-pregnanolone, respectively, while gray filled and open circles represent allopregnanolone and *ent*-allopregnanolone, respectively. Panels A, B, C, D and E show results for POPC, POPC:POPE:POPS=30:30:40, POPC:CHOL=70:30, POPC:Brain-SM:CHOL=35:35:30, and RBC ghosts. Phospholipid concentration is 50 μM.

glycolipids, phosphatidylinositolphosphates, and the presence of proteins including the cytoskeleton contribute to this observed difference.

3.4. Effects on phase transitions by differential scanning calorimetry

In a recent review, a mechanism for lipid-related effects in anesthesia was suggested to originate from the effects of anesthetics on the phase transition of phosphatidylserine, with consequent effects on the fusion of synaptic vesicles [62], and anesthetics seem to affect the release of synaptic transmitters [63–65]. A transient change in lipid phase occurs during the propagation of action potential [66,67], this change possibly representing a soliton [68]. Accordingly, it has been hypothesized that anesthetics may act by reducing the co-operativity of transitions and thus hinder action potentials [22]. To check for these possibilities, we tested the effect of steroid anesthetics (50 μM) on the main phase transitions of various 1.0 mM phospholipids (Tables 1–5). The steroid anesthetics had

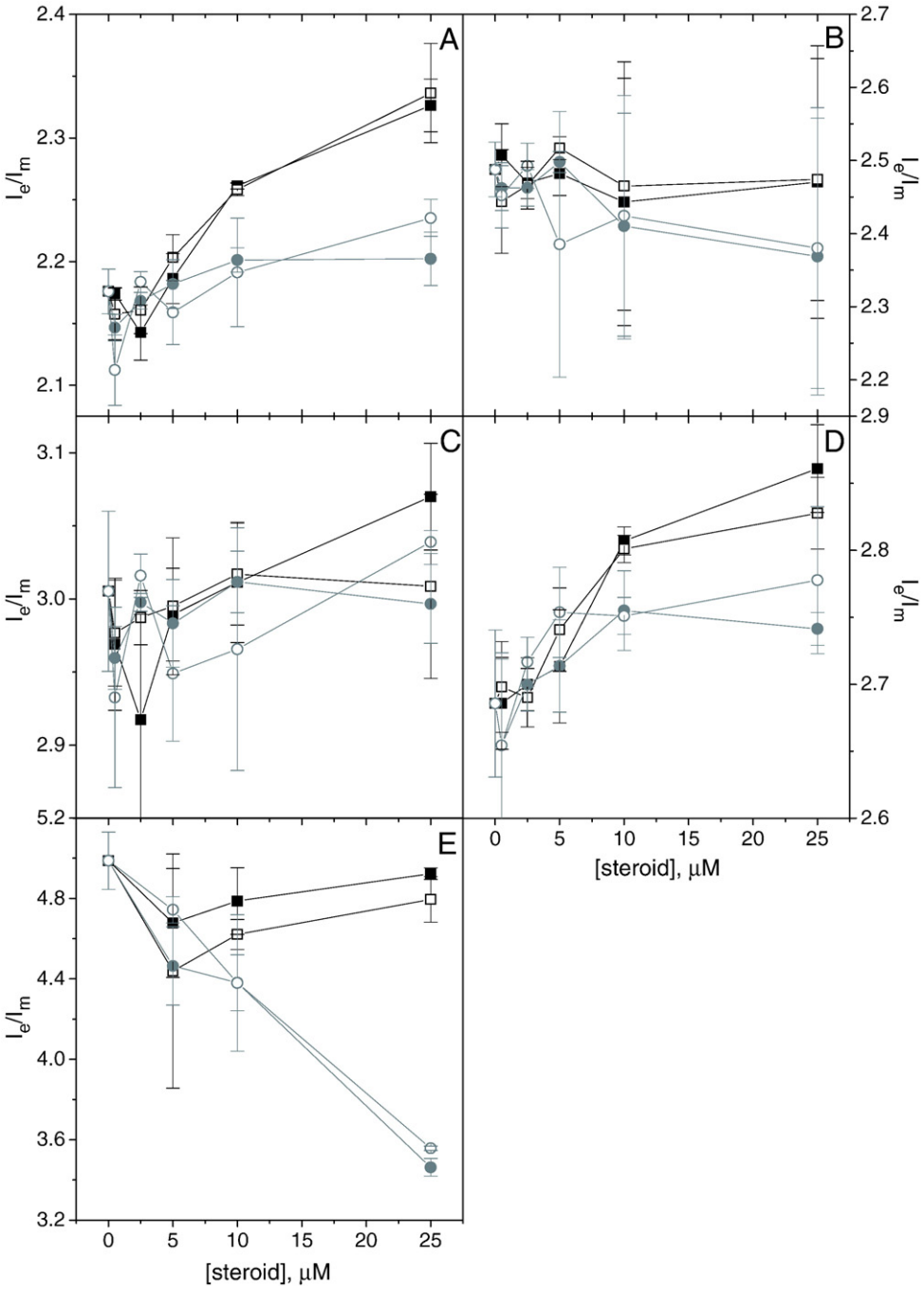

Fig. 8. The effects of steroids on I_e/I_m of bisPDPC in different bilayer matrices at 40 °C. Black filled squares and open squares represent pregnanolone and *ent*-pregnanolone, respectively, while gray filled and open circles represent allopregnanolone and *ent*-allopregnanolone, respectively. Panels A, B, C, D and E show results for POPC, POPC:POPE:POPS=30:30:40, POPC:CHOL=70:30, POPC:Brain-SM:CHOL=35:35:30, and RBC ghosts. Phospholipid concentration is 50 μM.

significant effects, and there were considerable differences between pregnanolones and allopregnanolones. However, no significant difference in the magnitude of effects could be seen between enantiomers. This suggests that the effects of the steroid anesthetics on phase transitions are not significant with respect to neurotransmitter release, action potential propagation and the mechanism of anesthesia in the clinical setting.

Though no difference is seen between the enantiomers, we nevertheless observe several quantitative and qualitative differences between the pregnanolones and the allopregnanolones. In the case of DPPC, both the pregnanolones and the allopregnanolones decrease pre and main transition temperature and cooperativity, but pregnanolones have a stronger effect (Table 1). Neither pregnanolones nor allopregnanolones appear to have a large effect on the phase behavior of brain-SM (Table 2).

Interestingly, in the case of DMPE we see very dramatic steroid effects. DMPE is known to display a high-temperature $L_c \rightarrow L_\alpha$ transition at 56.4 °C when DMPE has been incubated for several hours first at −50 °C and then at 47 °C [69,70]. In the absence of preincubation, we do not see this peak. Yet steroids, particularly

Table 1
Effects of steroid anesthetics on the phase transition of 1.0 mM DPPC, [steroid]=50 μM

DPPC	Pure	+PREG	+*ent*-PREG	+ALLOP.	+*ent*-ALLOP.
T_p, °C	34.67±0.07	26.8±2.0	27.305±0.007	30.7±0.3	28.9±2.5
FWHH_p, °C	1.52±0.03	10.2±2.1	10.12±0.02	4.2±0.2	7.1±3.6
ΔH_p, kJ/mol	6.22±0.01	3.8±1.5	3.41±0.05	4.2±0.3	4.2±0.6
T_m, °C	41.336±0.005	40.524±0.003	40.60±0.08	40.76±0.01	40.7±0.2
FWHH_m, °C	0.1582±0.0004	0.944±0.003	0.82±0.05	0.63±0.03	0.68±0.09
ΔH_{tot}, kJ/mol	43.4±1.3	44.6±0.2	40.2±4.4	43.1±2.5	41.2±1.4

Subscripts are as follows: p for pretransition, m for main transition, and tot for total. T_x, ΔH_x, and FWHH_x mark the temperature, enthalpy, and full width at half height, respectively, of transition *x*.

the allopregnanolones appear to induce the formation of L_c phase (Table 3, Fig. 9). The temperature for this transition coincides exactly with the transition temperature T_c from stable crystalline L_c phase into liquid-crystalline L_α phase. If we look at the average percentage of this second peak enthalpy of the total enthalpy, we get for pure DMPE 0.3±0.7%, and for samples with 5% PREG, *ent*-PREG, ALLOPREG and *ent*-ALLOPREG, we get 4.0±4.2, 6.6±6.4, 40.0±22.1 and 32.3±21.8%, respectively. Therefore, allopregnanolones appear to favor strongly the formation of the L_c phase by DMPE even without a cold incubation.

The steroids have only modest effects on the phase transition of 1 mM DMPS in the presence of 150 mM NaCl (Table 4), pregnanolones increasing FWHH and decreasing $T_{1/2}$ somewhat more than allopregnanolones. However, the situation is drastically different for DMPS in the absence of added salt (see Fig. 10). The results are summarized in Table 5. Interestingly, while in a DMPS matrix at low salt (with no added NaCl), 50 μM pregnanolones and allopregnanolones cause an equal decrease in $T_{1/2}$ the result is achieved by different means: pregnanolones decrease T_1 by almost 2.5 °C and possibly have a small decreasing effect on T_2 as well; whereas allopregnanolones decrease T_1 by only 0.5 °C and do not decrease T_2 at all, but instead cause a drastic change in the relative enthalpies of the two peaks, the second peak fading into a minor hump on the first peak (see Fig. 10).

4. Discussion

4.1. Membrane effects of pregnanolones and allopregnanolones

A closer look at the differences in membrane effects between allopregnanolones and pregnanolones reveals certain patterns. Pregnanolones decrease Ψ and GP more effectively at low temperatures indicating a larger interfacial free volume or better hydration. In contrast, allopregnanolones decrease Ψ and increase GP more effectively at high temperatures, indicating a smaller interfacial free volume or less hydration.

In contrast to the GP results, pregnanolones caused a larger increase in the I_e/I_m ratio of membranes. Allopregnanolones appear to have little effect on the packing of the membrane interior, while they decrease the interfacial hydration sensed by Laurdan. Our previous data [53] suggest that pregnanolone has relatively little effects on acyl chain mobility as probed by diphenylhexatriene (DPH) steady state anisotropy. Accordingly, it would appear that pregnanolones decrease the free volume of the membrane interior more than do allopregnanolones or that the effect of the allopregnanolone-induced free volume decrease on I_e/I_m is masked by increasing acyl chain order. Additionally, allopregnanolones have a stronger effect on the headgroup region, dehydrating the headgroup region, possibly by reducing free volume available for water in this region by occupying this volume, or by affecting water structure. Any actual, large condensing effect appears unlikely to explain the effect, as changes in I_e/I_m are small. These suggestions agree well with the temperature behavior of the dipole potential. The increased headgroup spacing of lipids at higher temperatures would favor the binding of allopregnanolones more strongly, and explain why the dipole potential decrease by allopregnanolones gets stronger relative to that by pregnanolones.

Interestingly, the effect of steroids on the phase transition of DMPS showed very different effects. While pregnanolones decreased the temperatures of both peaks, widened the peaks, and increased the relative intensity of the second peak, allopregnanolones did not cause a significant change in the peak width, had little effect on the peak temperatures, but considerably increased the low temperature peak compared to the high temperature peak (Fig. 10). Similar to the behavior characterized for DMPG, the two peaks in the endotherm of DMPS likely are not independent transitions between several different phases but rather reflect one continuous transition from gel to fluid phase with regions of different curvatures causing the two-peaked melting curve [71]. Accordingly, it seems that pregnanolones are greater perturbants of lipid packing, and that pregnanolones and allopregnanolones favor different membrane curvatures. These conclusions agree with Laurdan GP data and bisPDPC I_e/I_m data. It is likely the non-planar structure of pregnanolones induces defects into the interfacial region while

Table 2
Effects of steroid anesthetics on the phase transition of 1.0 mM bovine brain-SM, [steroid]=50 μM

Brain-SM	Pure	+PREG	+*ent*-PREG	+ALLOP.	+*ent*-ALLOP.
T_m, °C	35.3±1.7	37.2±0.2	37.3±0.3	36.8±0.4	36.6±0.3
FWHH_m, °C	12.08±0.12	13.4±0.5	13.35±0.11	14.5±0.9	14.0±0.5
ΔH_{tot}, kJ/mol	26.4±1.2	23.9±3.6	24.2±2.3	31.3±2.3	28.2±3.8
$T_{1/2}$, °C	34.3±0.4	34.1±0.3	34.1±0.6	33.6±1.0	33.7±0.6

Symbols as in Table 1. $T_{1/2}$=temperature at which half of the total enthalpy is reached.

Table 3
Effects of steroid anesthetics on the phase transitions of 1.0 mM DMPE, [steroid]=50 μM

DMPE	Pure	+PREG	+*ent*-PREG	+ALLOP.	+*ent*-ALLOP.
T_m, °C	49.92±0.09	49.27±0.11	49.19±0.17	48.92±0.12	48.93±0.14
FWHH_m, °C	0.37±0.10	0.9±0.3	1.1±0.2	0.77±0.09	0.89±0.09
ΔH_{tot}, kJ/mol	24.7±1.6	28.4±2.1	21.9±8.4	33.0±5.7	27.5±7.1
$L_c \rightarrow L_\alpha$ observed	maybe seen in one	in 3 samples, weak	in 3(−4) samples, (very) weak	in 4 samples, strong	in 4 samples, strong
T_c, °C	56.95(?)	56.38±0.11	56.5±0.4	56.58±0.06	56.61±0.04
FWHH_c, °C	N/A	1.25±0.17	0.8±0.4	1.02±0.03	0.96±0.04
$\Delta H_c/\Delta H_{tot}$, %	0.3±0.7	4.0±4.2	6.6±6.4	40.0±22.1	32.3±21.8

Symbols as in Table 1. Scans were made in quadruplicate. The question mark indicates that it is uncertain whether there is real peak or just an artefact of baseline.

the more planar backbone of allopregnanolones fits well into the structure. Additionally, as discussed above, allopregnanolones had a weaker effect on bisPDPC I_e/I_m than pregnanolones, and in the cases of POPC:POPE:POPS (30:30:40) bilayers and erythrocyte ghosts allopregnanolones even decreased I_e/I_m while a slight increase was seen for pregnanolones. This suggests that either pregnanolones more strongly reduce free volume in the acyl chain region or do this without an increase in mobility. One possibility that agrees with all the data is that pregnanolones, at the concentrations used, penetrate more into the bilayer thereby perturbing the acyl chain packing slightly, decreasing the intrabilayer free volume, increasing the headgroup region free volume (or decreasing it less in PC membranes) and thus favoring a more negative curvature. On the other hand, a higher fraction of bound allopregnanolone remains at the headgroup region, decreasing the headgroup region free volume more strongly, decreasing the intrabilayer free volume less or even increasing it, perturbing the acyl chain packing less, and favor a more positive curvature. This would explain why allopregnanolones decrease T_p, T_m and the cooperativities of pre and main transitions of DPPC bilayers less than pregnanolones do.

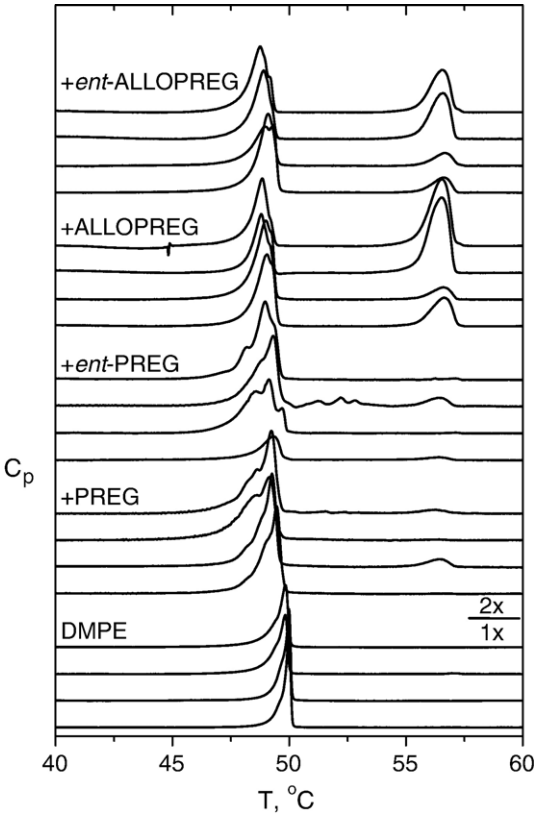

Fig. 9. Endotherms of 1 mM DMPE in 5 mM HEPES, 0.1 mM EDTA, pH 7.4. The lowest four curves are without steroids, the other sets contain indicated steroid at 50 μM concentration. The curves for steroid containing samples are shown with 2× magnification. Measurements were made in duplicate for two different DMPE lots, giving a total of four measurements.

The difference in the effects of allopregnanolones and pregnanolones on DMPE phase behavior could be explained, at least partly, by a greater perturbing effect of pregnanolones, thus making formation of the L_c phase less likely. However, this cannot explain why the steroids induce the formation of the L_c phase in the first place. One possibility is that this is connected to the hydration of DMPE headgroups. Gel phase DMPE tightly binds 6 water molecules/lipid, whereas stable, crystalline L_c phase binds only 2 water molecules/lipid and has strong inter-headgroup interactions [70]. Since steroid anesthetics are known to induce dehydration of proteins and lipids, and allopregnanolone would appear to induce greater dehydration as judged by Laurdan GP, a dehydrating effect of steroids appears as a possible explanation. Yet, it is remarkable that a mere 5% of steroids induce rapid transformation into the most stable phase, if only partially. It should also be noted that without steroids, 5 days of storage at cold temperature (−5 °C or 6 °C) does not cause this transformation [69]. In addition to the dehydration, steroid-induced softening effects or a local ordering of headgroups resembling the L_c phase may contribute to the formation of nuclei. However, more detailed examination of this phenomenon exceeds the scope of the present study.

4.2. Evidence against lipid-mediated actions by steroids

The well-known, strong diastereoselectivity of steroid interactions in lipid bilayers is evident as the behavior of pregnanolones and allopregnanolones show significant differences in many of the measured parameters. To compare the membrane mole fractions of the present study to those in anesthesia, we need to know the membrane molar partitioning coefficient K_p and the concentrations of the steroids and phospholipids in brain. First, while K_p for pregnanolone or allopregnanolone is not available, the values for similar steroids (such as progesterone and estrogens) range between

Table 4
Effects of steroid anesthetics on the phase transition of 1.0 mM DMPS, [steroid]=50 μM, [NaCl]=150 mM

DMPS	Pure	+PREG	+*ent*-PREG	+ALLOP.	+*ent*-ALLOP.
T_p, °C	13.08±0.04	13.14±0.07	13.15±0.09	12.97±0.09	12.97±0.17
FWHH_p, °C	1.8±1.0	1.15±0.07	1.06±0.09	1.8±0.9	1.6±0.3
ΔH_p, kJ/mol	0.34±0.09	0.45±0.08	0.55±0.02	0.19±0.07	0.37±0.13
T_m, °C	36.41±0.05	34.81±0.05	35.18±0.04	35.62±0.04	35.70±0.20
FWHH_m, °C	0.39±0.05	2.8±0.2	1.74±0.13	1.03±0.08	1.16±0.11
ΔH_{tot}, kJ/mol	32.2±0.3	31.9±0.9	32.2±2.0	29.4±1.7	32.5±0.4
$T_{1/2}$, °C	36.22±0.04	34.14±0.09	34.52±0.14	35.253±0.007	35.25±0.11

Symbols as in Table 1. $T_{1/2}$=temperature at which half of the total enthalpy is reached.

0.3 and 1.0×10^5 [72–74]. In a study where the anesthetic threshold level in rats was detected by EEG and the rats were subsequently dissected, it was found that the threshold concentration in the hippocampus and brain stem was approximately 22–34 μmol/kg for pregnanolone and 19–24 μmol/kg for allopregnanolone [75], suggesting that allopregnanolone is slightly more effective than pregnanolone. Contrary data with different assays have also been reported [13]. Yet, natural allopregnanolone and pregnanolone appear to have about equal anesthetic potency. Given that the total brain phospholipid of rats is approximately 46 mmol/kg [76], then for both the lowest and highest estimate of the K_p the mole fraction in brain plasma membranes at the anesthetic threshold would be approximately 1/2500–1/1400, whereas in our experimental systems the mole fraction in plasma membranes ranges approximately 1/3400–1/56 for the lowest estimate of K_p, and 1/1200–1/22 for the highest estimate of K_p. While these mole fractions give a rough guide, natural membranes contain domains with different lipid compositions, and the partitioning between domains is likely different, so the local mole fraction could fall outside this range. Uncertainties such as these, including the lack of information about the total relevant phospholipid concentration in experimental preparations such as encountered in tadpole assays or patch clamp recordings make it difficult to compare the potentially relevant membrane concentrations in different systems. Nonetheless, the ability of anesthetic steroids to potentiate responses at $GABA_AR$ of cultured neurons saturates at approximately 10 μM [13]. As some of the effects on phospholipids appear to saturate at similar concentrations, it is difficult to exclude lipid-mediated actions based on such arguments alone.

However, no evidence of significant enantioselectivity was observed with respect to any property studied. Based on the interpretation of data assigning different spontaneous curvatures to allopregnanolones and pregnanolones, but the same curvatures to enantiomers, the first integral moment of the lateral pressure profile should be equal for enantiomers. However, a 300% difference in anesthetic potency and 500% difference in effects on $GABA_AR$ by allopregnaolone enantiomers has been reported [13] and the enantiomers of allopregnanolone and pregnanolone have distinct effects on the function of the ρ_1 receptor [1]. Accordingly, these data provide strong support for the view that the anesthetic effects are mediated by direct binding to proteins in the case of steroid anesthetics. As similar though less extensive data exist on the membrane interactions of various enantiospecific anesthetics [13,14,19,38], it appears likely that this is common to anesthetics. At least the data place the burden of proof for a lipid-mediated hypothesis heavily in the hands of the advocates of this mechanism of anesthesia. Although anesthetics as lipid-soluble compounds have various effects on lipid bilayers, there is no evidence to suggest that these effects would lead to anesthesia. The most promising hypothesis of lipid-mediated anesthesia is the lateral pressure profile hypothesis [25,26]. There currently exists no method to measure the actual lateral pressure profile, and it is accessible only by calculations and simulations [25,26,77,78], although properties related to the first and second integral moments of lateral pressure profile can be assessed [79,80]. This profile can be very complex and specific to a compound [36]. To seal

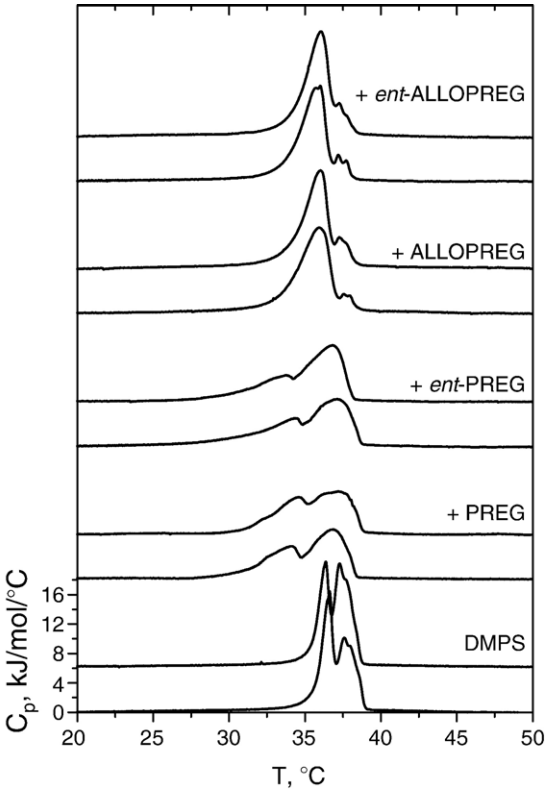

Fig. 10. Endotherms of 1 mM DMPS in 5 mM HEPES, 0.1 mM EDTA, pH 7.4. The lowest two curves are without steroids, the other pairs contain indicated steroid at 50 μM concentration. Spacing of values on *y*-axis is same for all curves, but for the sake of clarity scale is shown only for lowest curve.

Table 5
Effects of steroid anesthetics on the phase transition of DMPS, [steroid]=50 μM, [DMPS]=1.0 mM, [NaCl]=0 mM

DMPS	Pure	+PREG	+*ent*-PREG	+ALLOP.	+*ent*-ALLOP.
T_1, °C	36.5±0.2	34.3±0.3	34.0±0.5	35.97±0.09	35.99±0.03
T_2, °C	37.4±0.2	37.0±0.3	37.0±0.2	37.6±0.5	37.62±0.13
$T_{1/2}$, °C	36.8±0.2	35.5±0.2	35.37±0.12	35.55±0.13	35.5±0.2
FWHH_{tot}, °C	1.9±0.4	4.9±0.6	3.8±1.3	2.0±0.4	1.77±0.09
ΔH_{tot}, kJ/mol	34.9±1.0	30.42±0.11	32.5±1.1	38.8±4.8	36.2±4.0

Symbols as in Table 1. Subscripts 1 and 2 refer to first and second transition, respectively. $T_{1/2}$=temperature at which half of the total enthalpy is reached.

the debate, atomistic molecular dynamics simulations of the effects of anesthetic enantiomers of various classes of anesthetics on the lateral pressure profile are needed. In the case of steroid enantiomers, enantiospecific effects on the lateral pressure profile appear unlikely, as it is unlikely that the enantiomers of anesthetics would have a significantly different effect on lateral pressure profile and yet be identical in all their other effects on membranes. While many sources may contribute to the anesthetic potency, we may still conclude that it is unlikely that the lateral pressure profile is a major mechanism for general anesthesia in the case of anesthetic steroids. Rash generalizations should not be made, for not only is the spectrum of the affected proteins different but volatile anesthetics and steroid anesthetics are also clearly different in other respects [80].

4.3. Conclusion

We have described the interactions of two pairs of anesthetic steroid enantiomers with a range of lipid bilayers. Neither of the pairs shows any difference in any interaction with lipid bilayers, although anesthesia or actions at likely target proteins show enantioselectivity. This suggests that these interactions are not of primary importance for the anesthetic activity of steroid anesthetics. The 5α-steroids and 5β-steroids, despite their structural similarity, show great differences between pairs, seemingly in agreement with the suggestion that these steroid groups would have opposite actions on integral membrane proteins by changing membrane properties differently [2]. Our data proving the lack of enantiospecificity for steroid/lipid interactions of these steroids has allowed us to address the hypothesis of lipid-mediated action of steroids on $GABA_C\rho_1Rs$. As these steroid enantiomers were shown to affect $GABA_C\rho_1Rs$ enantiospecifically [1], we have shown that the hypothesis of Morris and Amin [2] is unlikely to be correct. Morris and Amin [2] showed that the critical amino acids in transmembrane segments 2 and 3 of $GABA_C\rho_1R$ required for the steroid effects are likely not binding sites. As these residues also are unlikely to be sensors of the lipid environment [1], most likely they and possibly similarly located critical amino acids on other ligand-gated ion channels are involved in the transduction of the binding signals from other sites on proteins. Accordingly, as previously suggested for cholesterol and its enantiomer [81], these two pairs and similar pairs of natural and *ent*-steroids will likely prove to be useful tools in studies of steroid actions on membrane proteins, as any significant enantiomeric specificity would appear to favor direct binding to proteins.

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