Effect of Ethanol on Metarhodopsin II Formation Is Potentiated by Phospholipid Polyunsaturation†

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ABSTRACT: The role of phospholipids in modulating the effect of ethanol on membrane receptor activation was investigated by studying the extent of metarhodopsin II (MII) formation in vesicles formed from POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and PDPC (1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine) and in native rod outer segment disk membranes as a function of ethanol concentration. The equilibrium concentration of MII, the G protein-activating form of photoactivated rhodopsin, was found to increase as a function of ethanol concentration in all three bilayers. Phospholipid composition had a marked effect on ethanol potency, with the presence of polyunsaturated phospholipid acyl chains increasing ethanol potency by 40%. The effects of ethanol on lipid acyl chain packing in POPC and PDPC were investigated using frequency domain anisotropy decay measurements of the fluorescent membrane probe 1,6-diphenyl-1,3,5-hexatriene. Enhanced formation of MII due to the presence of ethanol was correlated with the effects of ethanol on acyl chain packing properties. These findings support a phospholipid-mediated mechanism for the action of ethanol in modulating integral membrane receptor conformation.

The ability of alcohols and anesthetics to modify the activity of an extensive variety of integral membrane receptors and ion channels has been widely studied. Two generally opposing theories of the mechanism of alcohol and general anesthetic action have been advanced, one based on alteration of phospholipid bilayer properties by these agents and the other based on their direct interaction with membrane proteins (Miller, 1985; Franks & Lieb, 1994). The lipid bilayer-based mechanism dates from the observations of Meyer (1901) and Overton (1901) that anesthetic potency is directly correlated with solubility in olive oil. A recent modification of the lipid bilayer-mediated mechanism proposes disruption of the lipid–protein interface by alcohols and anesthetics (Fraser et al., 1990; Lopes & Louro, 1991; Abadji et al., 1993). The direct binding mechanism first gained widespread attention following experiments demonstrating that the activity of a soluble enzyme, firefly luciferase, could be inhibited by a diverse series of alcohols and anesthetics (Franks & Lieb, 1984). More recent studies have demonstrated that the effects of several anesthetics are stereospecific; that is, one stereoisomer has a 2–3 fold greater potency over its mirror image (Franks & Lieb, 1994). These results provide some of the strongest evidence supporting the modification of membrane protein activity due to the direct binding of anesthetic and/or ethanol to proteins. Although indirect evidence exists supporting the alternate hypothesis, the question of whether or not the alteration of phospholipid bilayer physical properties contributes to the action of general anesthetics and alcohols in modulating the functional activity of integral membrane proteins remains to be conclusively answered.

In general, it is difficult to separate changes in protein function due to direct drug–protein interaction from those due to drug-induced alteration of lipid–protein interactions. One way to delineate lipid bilayer involvement is to examine alcohol or anesthetic dose–response behavior as a function of lipid bilayer composition. In addition, the abundance of highly unsaturated phospholipids in the membranes of postsynaptic neurons, retinal rod outer segments, and other excitable cells [for a review, see Salem (1989)] underlines the importance of understanding the role these phospholipids might play in mediating the effects of ethanol and other lipid-soluble agents.

The visual pigment rhodopsin is one of the most well-characterized members of the superfamily of G protein-coupled membrane receptors. It is located in the vertebrate ROS[1] disk membrane and consists of seven membrane-spanning α-helices linked by hydrophilic domains. Rhodopsin's amino acid sequence (Hargrave et al., 1983) and two-dimensional projection structure are known (Schertler et al., 1993), and an assignment of the sequence to the projection structure has recently been proposed (Baldwin, 1993). Within a few milliseconds of photon absorption, rhodopsin exists in a metastable equilibrium between the photointermediates MI and MII, described by K_{eq}, where K_{eq} = [MII]/[MI] (Mathews et al., 1963). MII is identified as the agonist-bound form of the receptor, which binds and activates the visual G protein (Emeis et al., 1982; Kibelbek et al., 1991). Equilibrium MII concentration is known to depend on the bilayer lipid composition and falls with decreasing phospholipid acyl chain unsaturation (O'Brien et al., 1977; Wiedmann et al., 1988; Mitchell et al., 1992; Gibson & Brown, 1993) and an increasing level of bilayer cholesterol (Mitchell et al., 1990; Mitchell et al., 1992).

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[1] Abbreviations: MI, metarhodopsin I; MII, metarhodopsin II; DPH, 1,6-diphenyl-1,3,5-hexatriene; ROS, rod outer segment; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PDPC, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; DAPC, diarachidonoyl-*sn*-glycero-3-phosphocholine; DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DTPA, diethylenetriaminepentaacetic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); BHT, butylated hydroxytoluene; PKC, protein kinase C.

We have investigated the role of phospholipid polyunsaturation in mediating the action of ethanol on integral membrane protein conformation by comparing the effect of ethanol on MII formation in POPC and PDPC vesicles and in ROS disk membranes, whose phospholipids contain about 50% docosahexaenoyl acyl chains (Stone et al., 1979). In a separate set of measurements, the effect of ethanol on phospholipid acyl chain packing was assessed by measuring the fluorescence lifetime and dynamic anisotropy of the membrane probe DPH in POPC and PDPC vesicles as a function of ethanol concentration.

EXPERIMENTAL PROCEDURES

Sample Preparation. POPC and PDPC were purchased from Avanti Polar Lipids Inc. and used without further purification. Rhodopsin in rod outer segments was prepared from frozen bovine retinas (Lawson Inc., Lincoln, NE), solubilized in octyl glucoside, and purified via concanavalin A affinity chromatography (Litman, 1982). Large unilamellar vesicles consisting of lyophilized lipid and purified rhodopsin in a molar ratio of 100 to 1, respectively, were prepared using a dilution reconstitution method (Jackson & Litman, 1985). Concentrated vesicle stock solutions were stored at 4 °C in an isotonic, pH 7.0 buffer consisting of 10 mM PIPES, 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, and 50 μM DTPA (PIPES buffer). The total phospholipid content of the vesicles for each sample preparation was determined by phosphate analysis following the method of Bartlett (1959). BHT at a level of 1 molecule per 500 phospholipid molecules was added to lipid stock solutions prior to lyophilization to prevent lipid peroxidation. All samples were prepared and handled in an argon atmosphere.

MI ↔ MII Equilibrium Spectra. Deconvolved difference spectra of MI ↔ MII equilibrium mixtures were derived from a series of four absorbance spectra acquired at 20 °C with a Hewlett-Packard 8452A diode array spectrophotometer (0.2-s measuring time yielded <0.3% bleach by the measuring beam). Collected spectra include (1) the initial rhodopsin-containing vesicle suspension, (2) the suspension 3 s after a brief strobe flash which passed through a 520 ± 25 nm band-pass filter (15–20% bleach), (3) the suspension following the addition of 2 M hydroxylamine to yield a final concentration of 30 mM hydroxylamine, and (4) the suspension after complete bleaching of the sample. Corrected difference spectra consisting of the equilibrium mixture of MI and MII present 3 s after the bleaching flash were constructed by subtracting curve 1 from curve 2 and adding to this difference spectrum the spectrum of the bleached rhodopsin. Individual MI and MII spectra were deconvolved from the spectrum of their equilibrium mixture by nonlinear least squares estimation of the sum of two asymmetric Gaussian bands, one for MI and one for MII. Concentrations of MI and MII were calculated using published extinction coefficients (Applebury, 1984). Analysis of all data sets was checked for internal consistency by ascertaining that the derived concentration of bleached rhodopsin was within 5% of the sum of the derived MI and MII concentrations.

Frequency Domain Measurements of DPH Fluorescence. Phase shift and modulation measurements at 10–12 frequencies log spaced from 2 to 100 MHz were made with a GREG 9000 multiple-frequency phase-modulation dynamic spectrofluorometer (ISS). Excitation at 325 nm was provided by a helium–cadmium laser, and emission was monitored after passing through a 400-nm cutoff filter. For lifetime measurements the excitation polarizer was set perpendicular, and the emission polarizer was set at the magic angle (54.7°). All fluorescent lifetime measurements were made relative to a POPOP–absolute ethanol reference solution. Total fluorescence intensity decay was modeled as the sum of two discrete lifetimes: $I_{tot}(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$. For the purpose of comparing the fluorescent lifetime of DPH in different bilayers, the intensity-weighted average lifetime, $\langle\tau\rangle$, was calculated according to $\langle\tau\rangle = (\alpha_1\tau_1^2 + \alpha_2\tau_2^2)/(\alpha_1\tau_1 + \alpha_2\tau_2)$. For differential polarization measurements, the polarization dependence of the sample photomultiplier tube was measured and corrected at each frequency for each sample. Dynamic depolarization of DPH was characterized using a P2P4 model, which includes an explicit bimodal orientational distribution function, $f(\theta)$ (Straume & Litman, 1987a). The bimodal DPH orientational distribution function is defined by the width of the Gaussian distribution of the orientational probability, θ_g, and the fraction of the probe occupying the orientational probability maximum parallel to the bilayer normal, $f_\parallel$. DPH dynamic depolarization is fully characterized by $f(\theta)$ and the perpendicular rotational diffusion coefficient, $D_\perp$ (Straume & Litman, 1987a,b). Overall equilibrium ordering experienced by DPH was quantified by the fractional free volume parameter, f_v, which characterizes the volume available for probe reorientational motion in the anisotropic bilayer relative to that available in an unhindered, isotropic environment, and is defined by $f_v = 1/(2f(\theta)_{max})\int f(\theta)\sin(\theta)\,d\theta$.

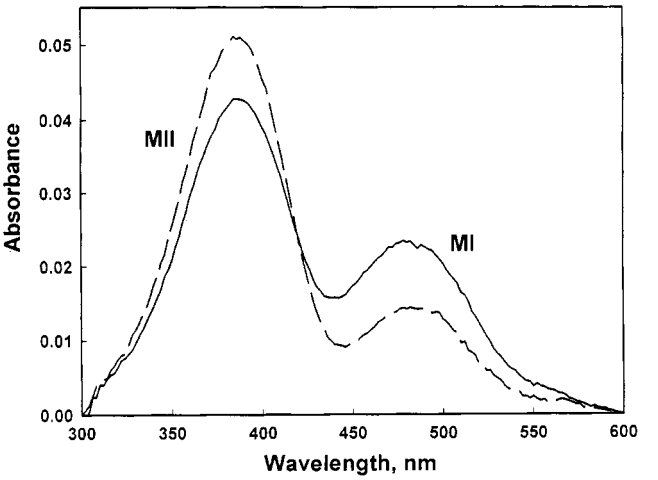

FIGURE 1: Example of corrected difference spectra of the MI ↔ MII equilibrium, showing the effect of ethanol. The curves show MI ↔ MII equilibrium spectra for rhodopsin in pH 7.5 PIPES buffer at 20 °C for rhodopsin in ROS disk membranes in the absence of ethanol (—) and in the presence of 0.5 M ethanol (- - -).

RESULTS

Effect of Ethanol on MI ↔ MII Equilibrium. The effect of ethanol on the activation of a G protein-coupled receptor was determined by monitoring MII formation in response to increased ethanol concentration. Ethanol increased the equilibrium concentration of MII at concentrations up to 1.5 M in ROS disk membranes and POPC and PDPC vesicles. An example of the ethanol-induced shift in the MI ↔ MII equilibrium toward MII is shown in Figure 1 for ROS disks in 0.5 M ethanol. In all three bilayers, neither the wavelength of maximum absorbance nor the overall shape of the MI and MII absorption bands was altered by ethanol.

Due to the strong dependence of K_{eq} on bilayer phospholipid composition, the effects of ethanol on K_{eq} in these three bilayers were expressed in terms of the additional change in free energy for MI ↔ MII, $\Delta(\Delta G)$, due to the presence of ethanol. The quantity $\Delta(\Delta G)$ is defined by $\Delta(\Delta G) = \Delta G_{+EtOH} - \Delta G_{-EtOH}$, where $\Delta G = -RT\ln(K_{eq})$ and ΔG_{+EtOH} and ΔG_{-EtOH} are the

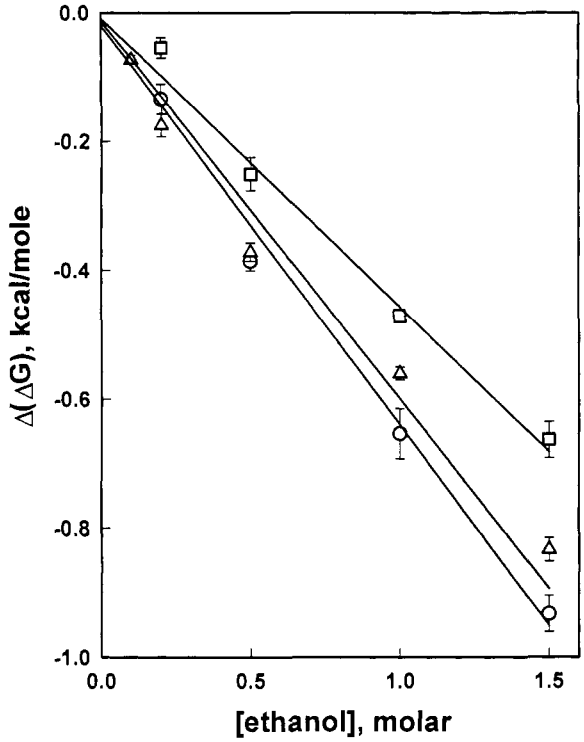

FIGURE 2: Dose–response for the effect of ethanol on the MI ↔ MII equilibrium for rhodopsin in POPC vesicles (□), PDPC vesicles (○), and ROS disk membranes (△). The ethanol-induced shift in the MI ↔ MII equilibrium is given in terms of $\Delta(\Delta G)$, as described in the text.

change in free energy in the presence and absence of ethanol, respectively. The effect of ethanol on the MI ↔ MII equilibrium for rhodopsin in each bilayer was determined by plotting $\Delta(\Delta G)$ against ethanol concentration. The resulting dose–response curves, shown in Figure 2, demonstrate that ethanol decreases $\Delta(\Delta G)$, i.e., increases the equilibrium MII concentration, in all three bilayer systems. Slopes of the dose–response lines are -0.44 ± 0.03, -0.62 ± 0.04, and -0.59 ± 0.03 kcal mol^{-1} [ethanol]$^{-1}$ for POPC, PDPC, and ROS disks, respectively. The smaller slope of the ethanol dose–response line for rhodopsin in POPC demonstrates that ethanol is about 40% less potent in a POPC bilayer than in a PDPC bilayer. The essentially identical effect of ethanol on rhodopsin in PDPC vesicles and ROS disks, despite their considerable differences in headgroup composition, is consistent with the similar content of docosahexaenoyl acyl chains for the two bilayers.

Effects of Ethanol on DPH Fluorescence Lifetime and Acyl Chain Packing. In order to characterize the effect of ethanol on acyl chain packing properties, the total intensity decay and anisotropy decay of DPH in large, unilamellar POPC and PDPC vesicles were measured in the presence of 0–1 M ethanol. All intensity decays were well-characterized by the sum of two exponentials ($\chi^2 > 1.2$; data not shown), and anisotropy decays were fit with the P2P4 model. In order to facilitate comparisons between the two bilayers and different ethanol concentrations, intensity decays were summarized by the intensity-weighted fluorescence lifetime, $\langle\tau\rangle$, and the P2P4 orientational distribution function was summarized by the fractional volume, f_v. In both bilayers, the ethanol-induced changes in these two parameters paralleled one another; that is, all ethanol concentrations that increased f_v also increased $\langle\tau\rangle$ (Figure 3). These parallel changes are unusual, because other factors, such as bilayer cholesterol content and temperature, produce opposite changes in f_v and $\langle\tau\rangle$. At low

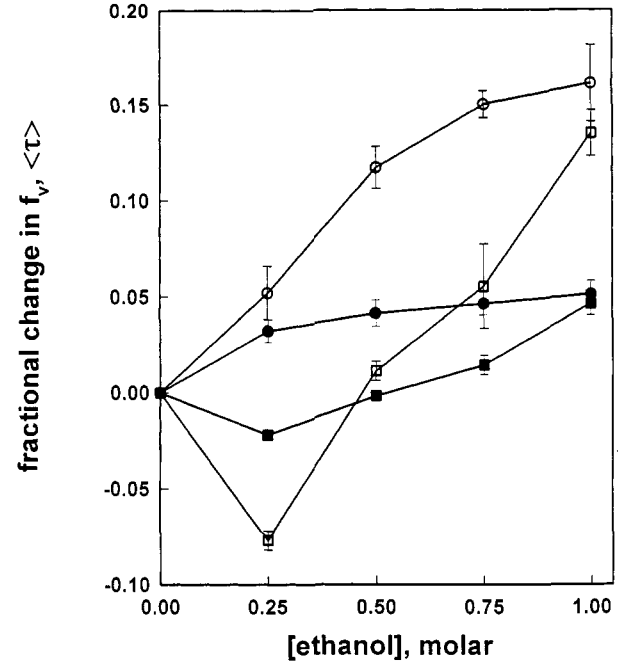

FIGURE 3: Effects of ethanol on DPH intensity-weighted average fluorescence lifetime, $\langle\tau\rangle$, in POPC (■) and PDPC (●) and DPH fractional free volume, f_v, in POPC (□) and PDPC (○). $\langle\tau\rangle$ and f_v are defined in the section on Frequency Domain Measurements of DPH Fluorescence in Experimental Procedures.

ethanol concentrations the ethanol- induced changes in both f_v and $\langle\tau\rangle$ are quite different for POPC and PDPC, with 0.25 M ethanol decreasing both f_v and $\langle\tau\rangle$ in POPC but increasing these parameters in PDPC. At 1 M ethanol the effects in POPC and PDPC are similar, with $\langle\tau\rangle$ being increased by about 5% and f_v by about 15% in both bilayers.

DISCUSSION

Evidence exists to support a direct binding mechanism for the action of ethanol on ligand-gated channels (Franks & Lieb, 1994). A recent study of PKC activity reported a dependence on bilayer composition of the effects of alcohols and anesthetics (Slater et al., 1993). However, inhibition of PKC activity by these agents was also observed for a lipid-free preparation. These results suggest a mixed mode of action for ethanol and anesthetics in this system. In the present study, certain sites of direct ethanol–rhodopsin binding can be eliminated. Ethanol cannot displace the ligand from its binding site in rhodopsin, since the agonist, *all-trans*-retinal, and antagonist, 11-*cis*-retinal, which are interconverted by light, are covalently bound to opsin. The absorption spectrum of rhodopsin is determined by the interaction of retinal with the amino acid side chains of opsin. Thus, binding of ethanol in the hydrophobic retinal binding domain would perturb the electronic environment of the chromophore, altering the absorption bands of MI, MII, and/or unbleached rhodopsin. However, ethanol had no effect on the peak positions or half-bandwidths of rhodopsin, MI, and MII in ROS disks, POPC, or PDPC. In addition, the molar extinction coefficients of all spectral species were unaffected by ethanol, as evidenced by consistent mass balance, i.e., [MI] + [MII] = [bleached rhodopsin], at all ethanol concentrations. The retinal binding pocket is thus eliminated as a possible site of ethanol interaction.

In both POPC and PDPC, ethanol concentrations above 0.25 M increased both $\langle\tau\rangle$ and f_v. This is in sharp contrast to other factors which perturb $\langle\tau\rangle$ and f_v. Changes in

temperature, bilayer cholesterol, and phospholipid unsaturation lower f_v while raising $\langle\tau\rangle$ (Straume & Litman, 1987a,b). The usual interpretation of these effects is that a more ordered acyl chain packing, consistent with decreased values of f_v, reduces the size and frequency of fluctuations in the phospholipid headgroup region (Straume & Litman, 1987a,b). This results in decreased water penetration and reduced quenching of DPH by water, yielding increased values of $\langle\tau\rangle$. Our data suggest that ethanol simultaneously produces looser acyl chain packing and reduced water penetration into the bilayer, a combination of effects unique to ethanol. The disparate effects of ethanol and cholesterol are consistent with their respective structures and with a binding site for both molecules in the glycerol backbone region. Binding in the glycerol backbone region by either molecule would reduce water penetration, accounting for higher values of $\langle\tau\rangle$. However, effects on acyl chain packing will differ, since the extended structure of cholesterol penetrates deep into the bilayer, ordering the acyl chains and lowering f_v, while the ethyl group of ethanol has little ability to penetrate the bilayer and order the phospholipid acyl chains. Therefore, ethanol can raise f_v by increasing the average headgroup spacing, allowing the acyl chains to disorder. This explanation is in good agreement with a recently proposed model for the binding of ethanol to phospholipid bilayers, based on deuterium NMR measurements, which places the major occupancy site of ethanol in the headgroup region, perhaps hydrogen bonded to a glycerol backbone carbonyl (Barry & Gawrisch, 1994), and an earlier model for the interaction of cholesterol with phosphatidylcholines (Huang, 1976).

The distinct effects of ethanol on f_v and K_{eq} in POPC and PDPC are consistent with a model of lipid–protein interaction, based on acyl chain packing free volume, whereby polyunsaturation increases bilayer permissiveness with respect to protein conformation changes (Mitchell et al., 1992). Analysis of the correlation between K_{eq} and f_v for purified rhodopsin incorporated into vesicles ranging from disaturated DMPC to dipolyunsaturated DAPC shows that MII formation is promoted by increasing f_v in a manner dependent on acyl chain unsaturation (Mitchell et al., 1992). Increased acyl chain unsaturation results in a greater increase in K_{eq} for a given increment in f_v. Examples of this type of correlation are shown in Figure 4A for egg PC and PDPC, and the slopes of the correlation lines for these two lipids are 8 K_{eq}/f_v and 30 K_{eq}/f_v, respectively. Points in Figure 4A were generated by changing temperature between 10 to 37 °C. Increasing the bilayer cholesterol levels in these systems reduces both K_{eq} and f_v and produces data points (not shown) which fall along the same correlation lines shown in Figure 4A (Mitchell et al., 1992, 1990). The correlations between ethanol-induced changes in K_{eq} and f_v for rhodopsin in POPC and PDPC are shown in Figure 4B. Whereas cholesterol induced a decrease in K_{eq} and f_v, ethanol increased both these parameters. The greater slope of the K_{eq} vs f_v correlation lines for PDPC demonstrates that changes in acyl chain packing due to either ethanol or cholesterol produce greater changes in K_{eq} in the polyunsaturated PDPC bilayer than in the more saturated POPC bilayer. Further evidence for the role of acyl chain unsaturation in modulating the effect of ethanol is provided by the similar dose–response behavior for rhodopsin in PDPC vesicles and ROS disks. PDPC vesicles and ROS disks contain similar average levels of docosahexaenoyl acyl chains; however, their headgroup compositions are very different, with disk membranes containing about 40% PC, 40% PE, and 15% PS (Stone et al., 1979). The identical effect of ethanol on

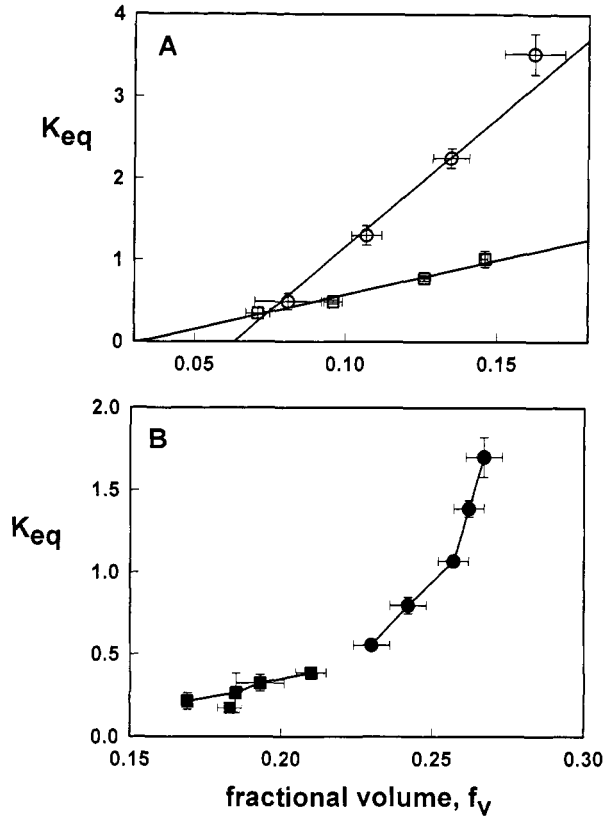

FIGURE 4: Effect of polyunsaturation on correlations between K_{eq} and f_v. (A) Example of correlation lines due to changes in temperature or bilayer cholesterol content for rhodopsin in PDPC (○) and egg PC (□). (B) Ethanol-induced changes in K_{eq} and f_v for rhodopsin in PDPC (●) and POPC (■). Values of f_v in panel B are higher than those in panel A because, in the present study, f_v was measured in pure lipid vesicles rather than in rhodopsin-containing vesicles.

rhodopsin in PDPC vesicles and ROS disk membranes (within experimental uncertainty) is consistent with the essentially identical composition of the *sn*-2 acyl chain between the two membranes and suggests that the mechanism whereby ethanol alters the MI ⇄ MII equilibrium is independent of headgroup composition.

Increasing ethanol concentration raises f_v and promotes formation of the G protein-activating conformation of rhodopsin, and this effect is magnified by increased phospholipid polyunsaturation. Thus, the effect of ethanol on MII formation may be understood in terms of the modulation of acyl chain packing free volume and the enhanced response in polyunsaturated bilayers. The differential effects of ethanol on rhodopsin in POPC and PDPC make it clear that whatever the precise molecular mechanism of ethanol's action on this integral membrane receptor, it is modulated by the lipid bilayer and is potentiated by acyl chain polyunsaturation. These results support a lipid-mediated mechanism for the action of ethanol on modulating a G protein-coupled receptor, rhodopsin. While there is evidence that the effect of ethanol on ligand-gated channels and some soluble proteins is via direct interaction with the proteins, no data exist to support this mode of action for integral membrane protein receptors, where a lipid-mediated mechanism may be the prevailing mode of action.

REFERENCES

Abadji, V. C., Raines, D. E., Watts, A., & Miller, K. W. (1993) *Biochim. Biophys. Acta 1147*, 143–153.

Applebury, M. L. (1984) *Vision Res. 24*, 1445–1454.

Baldwin, J. (1993) *EMBO J. 4*, 1693–1703.
Barry, J. A., & Gawrisch, K. (1994) *Biochemistry 33*, 8082–8088.
Emeis, D., Kuhn, H., Reichert, J., & Hofmann, K. P. (1982) *FEBS Lett. 143*, 29–34.
Franks, N. P., & Lieb, W. R. (1984) *Nature 310*, 599–601.
Franks, N. P., & Lieb, W. R. (1994) *Nature 367*, 607–614.
Fraser, D. M., Louro, S. R. W., Horvath, L. I., Miller, K. W., & Watts, A. (1990) *Biochemistry 29*, 2664–2669.
Gibson, N. J., & Brown, M. F. (1993) *Biochemistry 32*, 2438–2454.
Hargrave, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fong, S. L., Rao, J. K., & Argos, P. (1983) *Biophys. Struct. Mech. 9*, 235–244.
Huang, C.-H. (1976) *Nature 259*, 242–249.
Jackson, M. L., & Litman, B. J. (1985) *Biochim. Biophys. Acta 812*, 369–376.
Kibelbek, J., Mitchell, D. C., Beach, J. M., & Litman, B. J. (1991) *Biochemistry 30*, 6761–6768.
Litman, B. J. (1982) *Methods Enzymol. 81*, 150–153.
Lopes, C. M. B., & Louro, S. R. W. (1991) *Biochim. Biophys. Acta 1070*, 467–473.
Mathews, R., Hubbard, R., Brown, P., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215–222.
Meyer, H. (1901) *Arch. Exp. Pathol. Pharmakol. 46*, 338–346.
Miller, K. W. (1985) *Int. Rev. Neurobiol. 27*, 1–61.
Mitchell, D. C., Straume, M., Miller, J. L., & Litman, B. J. (1990) *Biochemistry 29*, 9143–9149.
Mitchell, D. C., Straume, M., & Litman, B. J. (1992) *Biochemistry 31*, 662–670.
O'Brien, D. F., Costa, L. F., & Ott, R. A. (1977) *Biochemistry 16*, 1295–1303.
Overton, E. (1901) in *Studien uber der Narkose*, Fischer, Jena.
Salem, N., Jr. (1989) in *New Protective Roles for Selected Nutrients* (Spiller, G. A., & Scala, J., Eds.) pp 109–228, Alan R. Liss Inc., New York.
Schertler, G. F. X., Villa, C., & Henderson, R. (1993) *Nature 362*, 770–772.
Slater, S. J., Cox, K. J. A., Lombardi, J. V., Ho, C., Kelly, M. B., Rubin, E., & Stubbs, C. D. (1993) *Nature 364*, 82–84.
Stone, W. L., Farnsworth, C. C., & Dratz, E. A. (1979) *Exp. Eye Res. 28*, 387–393.
Straume, M., & Litman, B. J. (1987a) *Biochemistry 26*, 5121–5126.
Straume, M., & Litman, B. J. (1987b) *Biochemistry 26*, 5113–5120.
Wiedmann, T. S., Pates, R. D., Beach, J. M., Salmon, A., & Brown, M. F. (1988) *Biochemistry 27*, 6469–6474.