# Communication

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# Primary Alcohols Modulate the Activation of the G Protein-coupled Receptor Rhodopsin by a Lipid-mediated Mechanism*

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**The visual pigment rhodopsin is a prototypical member of the G protein-coupled receptor superfamily. In this study, we have investigated the effect of a series of *n*-alcohols on the formation of metarhodopsin II (MII), the photoactivated conformation of rhodopsin, which binds and activates transducin. When rhodopsin was photolyzed in the presence of several *n*-alcohols, increased MII formation was observed in the order ethanol > butanol > hexanol, whereas longer chain *n*-alcohols inhibited MII formation with decanol > octanol. The magnitude of the stimulatory effects was greater in a more highly unsaturated phospholipid. Alcohols, which enhanced MII formation also increased phospholipid acyl chain packing free volume, while those that decreased this bilayer property inhibited MII formation. An apparent discontinuity in the effect of these alcohols results when their potency is calculated in terms of the total aqueous alcohol concentration. In sharp contrast, a continuous variation in their behavior is observed, when their potency is calculated in terms of the amount of alcohol partitioned in the membrane. Our findings strongly support a lipid-mediated mechanism of action for alcohols on rhodopsin and, by analogy, for other G protein-coupled receptors.**

The mechanism of action of alcohols and general anesthetics is generally discussed in terms of two opposing mechanisms. The first involves an alteration of phospholipid bilayer properties by these agents, resulting in a modulation of membrane protein function, while the second is based on the direct interaction with membrane proteins (1, 2). The lipid mechanism was based originally on the observation (3, 4) that anesthetic potency correlated directly with the solubility of an anesthetic in olive oil. Additional support for the lipid mechanism came from the observation that acute exposure of membranes to ethanol resulted in a disordering of the phospholipid acyl chain packing (5). Subsequent experiments demonstrating that the activity of a soluble enzyme, firefly luciferase, could be inhibited by a diverse group of alcohols and general anesthetics (6) have focused attention on the protein binding hypothesis.

Many of the recent studies aimed at elucidating the mechanism of action of alcohols and general anesthetics have dealt with the effect of these agents on ligand-gated channels, whose ligand binding site is in a portion of the protein external to the bilayer (1, 7–10). The superfamily of G protein-coupled receptors has received relatively little study. In these receptors, the ligand binding sites are formed by their transmembrane helical segments and lie at the median point of the bilayer. One of the best characterized members of this superfamily is rhodopsin, which triggers the visual transduction pathway in rod cells. A metastable equilibrium between MI[1] and MII (where $K_{eq}$ = [MII]/[MI]) is established within milliseconds of photon absorption (11). MII binds and activates the visual G protein, transducin (12, 13). MII formation increases with higher levels of phospholipid acyl chain unsaturation (14–17) and decreasing levels of cholesterol (16, 18).

Differentiating between the two proposed mechanisms of action of alcohols and general anesthetics is difficult. However, studying the lipid dependence of the dose-response behavior of receptors in reconstituted systems is an explicit way of determining the lipid involvement in the action of these molecules. The membranes of postsynaptic neurons, retinal rod outer segments, and other excitable cells contain a preponderance of highly unsaturated phospholipids (for a review, see Ref. 19), suggesting a potential role for these phospholipids in mediating the effects of ethanol and other lipid-soluble agents. Recently, we published studies which support the involvement of a lipid mechanism in the enhancement of MII formation by ethanol (20). Variable chain length alcohols have been employed extensively as models for general anesthetics (1, 2). To further examine the involvement of lipids in the action of ethanol and general anesthetics, we have studied the effect of several *n*-alcohols on MII formation in ROS disks, PDPC, and POPC vesicles. In addition, the time-resolved anisotropy decay of the hydrophobic membrane probe, DPH, was used to characterize changes in phospholipid acyl chain packing properties in response to the addition of these alcohols.

## EXPERIMENTAL PROCEDURES

*Sample Preparation*—Intact ROS disks were prepared from frozen bovine retinas as described previously (21). Samples for all studies were in a low salt buffer (10 mM HEPES, 50 μM diethyltriaminepentaacetic acid, pH 7.5; HNS buffer) and all measurements were made at 20 °C. Suspensions of intact ROS disks (8 μM rhodopsin) were extruded 10x through a 0.2-μm pore filter to reduce light scattering. Rhodopsin was purified and reconstituted with defined phospholipids to yield rhodopsin-containing large unilammelar vesicles as described previously (22, 23). The phospholipids PDPC and POPC were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The effect of the *n*-alcohols on $\Delta G$ for the MI ↔ MII equilibrium at each concentration was determined according to $\Delta(\Delta G) = \Delta G_{+alcohol} - \Delta G_{-alcohol}$, where $\Delta G_{-alcohol} = -RT\ln(K_{eq,-alcohol})$, where $R$ is the gas constant, and $T$ is the absolute temperature. Bulk alcohol concentrations varied as follows: ethanol: 0.15–1.5 M; butanol: 9–108 mM; hexanol: 1.16–7 mM; octanol: 0.25–0.98 mM; and decanol: 42–170 μM. Bilayer mole fractions, $\chi_{alcohol}$, of *n*-alcohols were calculated from

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[1] The abbreviations used are: MI, metarhodopsin I; MII, metarhodopsin II; ROS, rod outer segment; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PDPC, 1-palmitoyl-2-docosahexaenoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene.

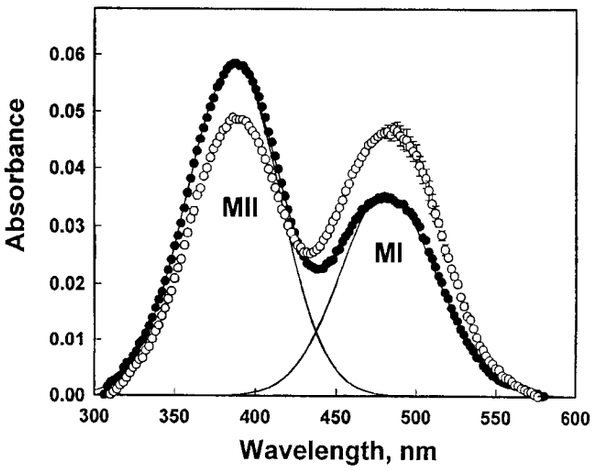


FIG. 1. **Difference spectra, corrected for the presence of unbleached rhodopsin, of the MI-MII equilibrium for ROS disks (○) and ROS disks plus 40 mM butanol (●), in pH 7.5 HNS buffer at 20 °C.** The *solid curves* are the deconvolved MI and MII bands for ROS disks plus 40 mM butanol.

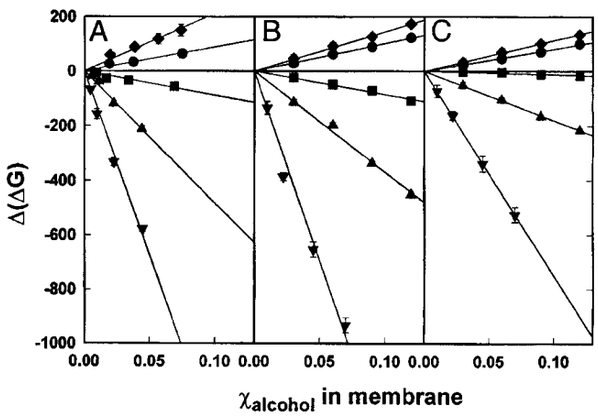


FIG. 2. **Dose-response curves for the effect of ethanol (▼), butanol (▲), hexanol (■), octanol (●), and decanol (♦) on the MI-MII equilibrium of rhodopsin in ROS disks (A), PDPC vesicles (*B*), and POPC vesicles (*C*).** Δ(Δ*G*) has the dimensions of cal/mol.

published membrane-buffer partition coefficients, *K*, determined for the partitioning of the *n*-alcohols into L-α-dimyristoylphosphatidylcholine (24) according to K = (mole fraction of alcohol in the lipid phase)/(mole fraction of alcohol in the water phase), and $\chi_{alcohol}$ = (moles alcohol in bilayer)/(moles alcohol in bilayer + moles lipid).

*Absorbance Spectroscopy*—Deconvolved difference spectra of MI ↔ MII equilibrium mixtures were derived from absorbance spectra acquired with a Hewlett-Packard 8452A diode array spectrophotometer (0.2-s measurements yielded <0.3% bleach by measuring beam) (25). Individual MI and MII bands were resolved by using a nonlinear least squares routine to fit the sum of two asymmetric Gaussian bands to difference spectra that had been corrected for the presence of unbleached rhodopsin (25). Concentrations were calculated using extinction coefficients of 44,000 $cm^{-1}$ $M^{-1}$ for MI and 38,000 $cm^{-1}$ $M^{-1}$ for MII (26). $K_{eq}$ is defined as [MII]/[MI].

*Time-resolved Fluorescence Measurements and Analysis*—ROS disks were suspended in HNS buffer at a rhodopsin concentration of 2.0 μM (~150 μM phospholipid) 0.5 μl of concentrated DPH in THF was added to give a final phospholipid to DPH ratio of 300. Decays of total fluorescence intensity and fluorescence anisotropy were measured with a K2 multifrequency cross-correlation phase and modulation fluorimeter (ISS, Champaign, IL) with excitation provided by an Innova 307 argon-ion laser (Coherent, Palo Alto, CA). Total fluorescence intensity decays were measured at 12 frequencies, logarithmically spaced from 5 to 250 Mhz, using magic angle polarization. Differential polarization measurements were made at 15 frequencies, logarithmically spaced from 10 to 300 Mhz. Total intensity decays were analyzed in terms of the sum of three discrete lifetimes. Anisotropy decays of DPH were characterized using a P2–P4 model, which included an explicit bimodal orientational distribution function, $f(\theta)$ (27). DPH dynamic depolarization is fully characterized by $f(\theta)$, the perpendicular rotational diffusion coefficient, $D_\perp$, and the anisotropy at time 0, $r_0$ (27, 28). Overall equilibrium ordering experienced by DPH was quantified by the 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 $f_v = 1/(2f(\theta)_{max})\int f(\theta)\sin(\theta)\,d\theta$.

## RESULTS

Rhodopsin is uniquely well suited for studies of the mechanism of action of anesthetics, since the ligand, retinal, is covalently bound to the receptor and acts as a reporter of receptor conformation. In addition, the retinal absorbance is very sensitive to the position of the amino acids in the retinal binding site, and the presence of any of the alcohols in the retinal binding pocket would be readily detected as a shift in the retinal absorption spectra. In our studies none of the alcohols altered the shape or location of the MI, MII, or rhodopsin absorption bands (Fig. 1), indicating that the alcohols did not partition into the retinal binding site.

The effect of various alcohols was characterized by the incremental change in Gibbs free energy, Δ(Δ*G*) (see "Experimental Procedures"), for the MI-MII equilibrium that resulted from the addition of the alcohol. All five alcohols altered the MI-MII equilibrium in a dose-dependent manner, producing unique linear correlations between Δ(Δ*G*) and the mole fraction of alcohol in the membrane, $\chi_{alcohol}$, Fig. 2. Ethanol, butanol, and hexanol increased the equilibrium concentration of MII, as shown by the negative slopes of their dose-response lines, while octanol and decanol decreased MII formation.

The behavior of the alcohols relative to MII formation in disks (Fig. 2*A*) and reconstituted vesicles (Fig. 2, *B* and *C*) was qualitatively similar. In these studies, we have defined the potency as the slope of the dose-response lines shown in Fig. 2. As shown in Fig. 3*C*, the potency of each of the 5 alcohols in the two reconstituted systems was quantitatively quite different, when compared with the disk (Fig. 3*B*). The effect of the excitatory alcohols in POPC dropped from 61% to 14% of the disk response going from ethanol to hexanol, whereas the response in PDPC was approximately equal to the disk. The similarity in behavior of rhodopsin in disks and PDPC is consistent with the phospholipid acyl chain composition of the two bilayers, which is about 50% 22:6*n*−3 (29). Figs. 3, *A* and *B*, compare the variation in potency of the alcohols, when their effect on MII formation is correlated with the total alcohol concentration, $M_{alcohol}$ and $\chi_{alcohol}$, respectively.

The effect of the alcohols on the bilayer phospholipid acyl chain packing properties was characterized by measuring the change in dynamic anisotropy of DPH in response to the addition of ethanol, butanol, hexanol, and decanol to disk membranes. The equilibrium orientational distribution properties of DPH are summarized by the derived parameter $f_v$. We have shown previously that $K_{eq}$ increases with increasing $f_v$ (16, 18, 30). In disk membranes, ethanol, butanol, and hexanol caused a concentration dependent increase in $f_v$, while decanol induced a decrease in $f_v$, Fig. 4. $K_{eq}$ *versus* $f_v$ was plotted for several values of $\chi_{alcohol}$ in the disk membrane. These data show that the variation in $K_{eq}$ induced by the alcohols is linearly related to the change in $f_v$ induced by the same alcohol, Fig. 5.

## DISCUSSION

Our previous studies demonstrate that increased MII formation is correlated directly with increased acyl chain packing free volume, as reported by the parameter $f_v$ (16, 18, 30) in a

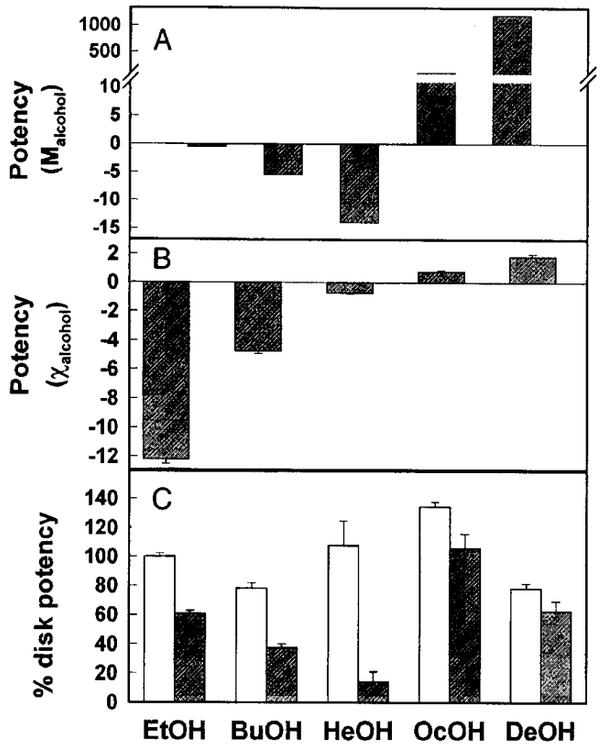


FIG. 3. **Potency of the *n*-alcohols relative to their effect on the MI-MII equilibrium.** *A*, potency for rhodopsin in ROS disks based on bulk alcohol concentration. Potency, calculated from the slope of $\Delta(\Delta G)$ *versus* $M_{alcohol}$ correlation lines, has units of Kcal/mol/$mM_{alcohol}$. *B*, potency for rhodopsin in ROS disks based on mole fraction of alcohol in the bilayer. Potency, defined as the slope of the dose-response lines shown in Fig. 2, has units of Kcal/mol/$\chi_{alcohol}$. *C*, a comparison of the potency of *n*-alcohols in PDPC (*open bars*) and POPC (*shaded bars*) vesicles relative to ROS disks shows that acyl chain composition modulates response. Percent of disk potency is given by [(potency$_{lipid}$/potency$_{disk}$) × 100].

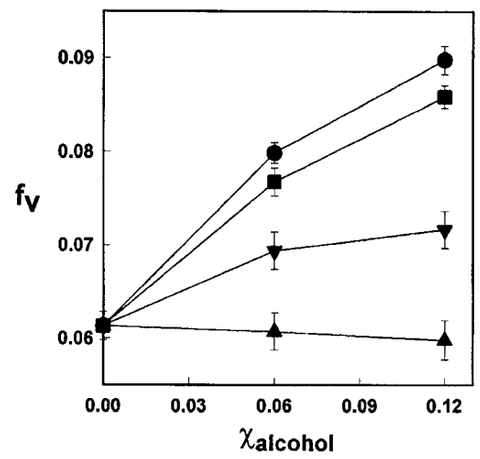


FIG. 4. **The effect of several *n*-alcohols, ethanol (▼), butanol (▲), hexanol (■),and decanol (♦) on the phospholipid acyl chain packing properties in the ROS disk membrane at 20 °C, as reported by the parameter $f_v$.** Alcohols that enhance MII formation also increase $f_v$, while decanol, which decreases MII formation also decreases $f_v$.

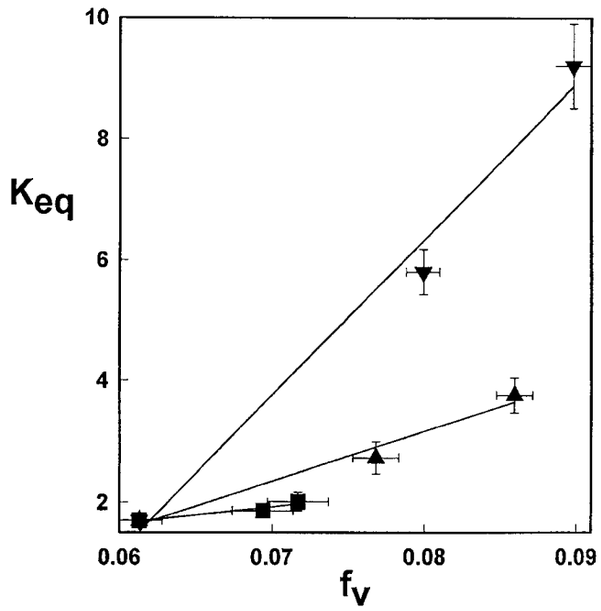


FIG. 5. **The change in $K_{eq}$ for MI-MII induced by ethanol (▼), butanol (▲), and hexanol (■) for rhodopsin in ROS disks at 20 °C is directly proportional to the concomitant change in $f_v$, demonstrating a linear correlation between $K_{eq}$ and alcohol-induced changes in phospholipid acyl chain packing properties.**

manner that depends upon acyl chain composition. Ethanol is reported to bind in the interfacial region of the bilayer, presumably hydrogen-bonded to the carbonyl oxygen of the glycerol backbone (31). The localization of ethanol in the interfacial region and the positive correlation between MII production and acyl chain packing free volume (16, 18, 30) provide a context for interpreting the disparate effects of the various alcohols studied here. Ethanol binding in the interfacial region is reported to increase the average head group spacing. This should increase the bilayer acyl chain packing free volume, and MII formation, which is consistent with our observations shown in Figs. 4 and 2, respectively. As the alcohol chain length increases, it progressively occupies more space behind the head group, reducing the gain in bilayer free volume associated with the increased head group spacing. For chain lengths greater than hexanol, the result is a net loss in bilayer free volume, Fig. 4, resulting in an inhibition of MII formation, Fig. 2. The effect of free volume on MII formation is consistent with the observation that the formation of MII is associated with an increase in volume of about 100 ml/mol, suggesting that MII has an expanded molecular volume relative to MI (32). Our data indicate that alcohols that promote MII formation disorder acyl chain packing, as reflected by increasing $f_v$, whereas those alcohols that inhibit MII formation order acyl chain packing and decrease $f_v$. Similar results were observed to differentiate anesthetic and nonanesthetic alcohols when the lipid acyl chain order parameter was plotted against $\chi_{alcohol}$ in nicotinic acetylcholine receptor-rich membranes (33). All anesthetic alcohols decreased acyl chain order, whereas nonanesthetic alcohols increased the acyl chain order parameter. The exact mechanism whereby higher levels of phospholipid acyl chain unsaturation amplifies the effect of the *n*-alcohols relative to MII formation, as shown in Fig. 3*C*, is not yet determined. However, possible explanations are either that the partition coefficients for the alcohols change with the acyl chain composition or that at equivalent $\chi_{alcohol}$, the acyl chain packing of polyunsaturated phosphatidylcholine is more readily perturbed than that of the monounsaturated phosphatidylcholine.

Upon going from hexanol to octanol, the receptor response changes from excitatory to inhibitory. When potency is given in

terms of $M_{alcohol}$, this transition appears as a sharp discontinuity, Fig. 3*A*. However, when potency is stated in terms of $\chi_{alcohol}$, this transition is seen to be part of a continuous decline in potency of the alcohols to promote MII formation. Thus the apparent discontinuity in behavior between hexanol and octanol is an artifact of the manner in which the data is expressed, *i.e.* correlating the response with $M_{alcohol}$ rather than $\chi_{alcohol}$ and is not an intrinsic system property. Although cutoff points have been described in certain systems, the class of receptors represented by rhodopsin does not exhibit this mode of behavior. These findings demonstrate that the manner in which the alcohol concentration is expressed can produce an apparent cutoff effect and reverse the order of potency of the alcohols. Clearly, in studies of the molecular mechanism of action of these alcohols, potency has more relevance if it is based on the concentration of alcohol at the site of action rather than on the total alcohol concentration present in the sample. Our results unequivocally demonstrate that the potency of the excitatory alcohols, relative to promoting increased MII formation, is inversely related to the aqueous-lipid partitioning, with ethanol having the highest potency and hexanol being the least potent.

While there is evidence to support the action of alcohols directly on ligand-gated ion channels (2, 7) and some peripheral membrane proteins (34), no such mechanism has been established for liganded receptors of the type studied here. Our results provide strong evidence that alcohols can modulate the formation of the G protein-activating conformation of rhodopsin, MII, by a lipid-mediated mechanism. By analogy, one would expect other G protein-coupled receptors, such as those that mediate the action of several neurotransmitters, to be sensitive to the presence of alcohols in a manner similar to rhodopsin. Neuronal and retinal tissue are rich in highly polyunsaturated phospholipid acyl chains, such as 22:6$n-3$ (19), which are more sensitive to the effects of alcohols. Thus, our findings suggest that the receptors in the membranes of neurons and photoreceptors will be particularly sensitive to the effects of alcohols and other lipid soluble agents.

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