Effect of Membrane Lipid Composition on the Conformational Equilibria of the Nicotinic Acetylcholine Receptor*

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The effects of cholesterol (Chol) and an anionic lipid, dioleoylphosphatidic acid (DOPA) on the conformational equilibria of the nicotinic acetylcholine receptor (nAChR) have been investigated using Fourier transform infrared difference spectroscopy. The difference between spectra recorded in the presence and absence of agonist from the nAChR reconstituted into 3:1:1 egg phosphatidylcholine (EPC)/DOPA/Chol membranes exhibits positive and negative bands that serve as markers of the structural changes associated with the resting to desensitized conformational change. These markers are absent in similar difference spectra recorded from the nAChR reconstituted into EPC membranes lacking both Chol and DOPA, indicating that the nAChR cannot undergo conformational change in response to agonist binding. When low levels of either Chol or DOPA up to 25 mol % of the total lipid are included in the EPC membranes, the markers suggest the predominant stabilization of a conformation that is a structural intermediate between the resting and desensitized states. At higher levels of either Chol or DOPA, the nAChR is stabilized in a conformation that is capable of undergoing agonist-induced desensitization, although DOPA appears to be required for the nAChR to adopt a conformation fully equivalent to that found in native and 3:1:1 EPC/DOPA/Chol membranes. The ability of these two structurally diverse lipids, as well as others (Ryan, S. E., Demers, C. N., Chew, J. P., Baenziger, J. E. (1996) J. Biol. Chem. 271, 24590–24597), to modulate the functional state of the nAChR suggests that lipids act on the nAChR via an indirect effect on some physical property of the lipid bilayer. The data also suggest that anionic lipids are essential to stabilize a fully functional nAChR. We propose that membrane fluidity modulates the relative populations of nAChRs in the resting and desensitized states but that subtle structural changes in the presence of anionic lipids are essential for full activity.

The nicotinic acetylcholine receptor (nAChR)† from Torpedo is a large multisubunit integral membrane protein that has been used extensively as a model for studying the mechanisms of lipid-protein interactions (1, 2). In native membranes, the nAChR transiently gates open a cation-selective ion channel across the postsynaptic membrane in response to the binding of agonists such as acetylcholine and carbamylcholine (Carb). Prolonged exposure to either agonist or a variety of noncompetitive antagonists leads to the stabilization of a channel inactive/desensitized (D) state. In reconstituted membranes, the ability of the nAChR both to conduct cations across the membrane and to undergo the resting to desensitized (R→D) conformational transition is highly sensitive to the composition of the surrounding lipid membrane. The molecular details of how lipids modulate the ability of the nAChR to undergo agonist-induced conformational change, however, remain unclear.

The original studies of Fong and McNamee (3) suggested that although the nAChR reconstituted into a dioleoylphosphatidylcholine (DOPC) membrane is not functional, the addition of both cholesterol (Chol) and an anionic lipid, such as dioleoylphosphatidic acid (DOPA), to the reconstituted DOPC membrane restores the ability of the nAChR both to conduct cations and undergo agonist-induced desensitization. The recovery of function in the presence of Chol and DOPA was attributed to both the formation of a lipid bilayer with an optimal membrane fluidity and a specific structural requirement of the nAChR for each lipid. The latter was proposed to result from the binding of each to distinct sites on the nAChR with the consequent formation of specific secondary structural features (3, 5–7).

Subsequent work has led to contradictory conclusions regarding the additional lipids that are required in a reconstituted DOPC membrane for the nAChR to adopt a functional conformation. McCarthy and Moore (8) proposed that anionic lipids are sufficient to stabilize a functional nAChR based on chemical labeling and a-bungarotoxin rate binding studies of the nAChR in membranes composed of egg phosphatidylcholine (EPC) and DOPA. Their work also found that when reconstituted into membranes composed of either EPC/Chol or EPC alone the nAChR adopts a predominantly D conformation (8). In contrast, the binding kinetics of ethidium bromide suggest that mixtures of DOPA/Chol support a functional nAChR whereas in either DOPC/DOPA or DOPC alone the nAChR is essentially “locked” in an R conformation (9; see also Ref. 10). Fourier transform infrared (FTIR) difference spectra are consistent with the data of McCarthy and Moore (8) in that they suggest that the nAChR in EPC alone is desensitized (11). In contrast to both studies, the FTIR data show that the presence of small amounts of either neutral or anionic lipids in an EPC membrane is sufficient to stabilize a fraction of the nAChRs in a functional conformation that is capable of undergoing agonist-induced conformational change. The FTIR data led to the suggestion that lipids modulate the relative number of nAChRs in the R and D states in the absence of agonist. The apparent ability of a variety of structurally diverse neutral and anionic lipids to modulate nAChR conformational change has not been satisfactorily accounted for.

The abbreviations used are: nAChR, nicotinic acetylcholine receptor; Carb, carbamylcholine; D, desensitized; R, resting; DOPA, dioleoylphosphatic acid; EPC, egg phosphatidylcholine; FTIR, Fourier transform infrared; ATR, attenuated total reflectance.

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lipids to modulate the equilibrium between the R and D conformations suggests further that lipids influence nAChR conformational equilibria via an indirect effect on some physical property of the membrane. Mixtures of a variety of structurally diverse neutral and anionic lipids in DOPC membranes all support nAChR cation flux (12). Recent FTIR studies have also been unable to detect any of the changes in nAChR secondary structure which were reported previously in the presence of neutral and/or anionic lipids (13).

The contradictory conclusions in the literature regarding the specific lipid requirements of the nAChR may reflect a variety of factors including the fact that the functional status of the nAChR in most studies has been assessed in reconstituted DOPC or EPC membranes with the additional lipid of interest found at a single molar percentage of the total membrane lipids (usually 25% or less). In many cases, functional data have also been interpreted in terms of the stabilization of either a fully functional or a nonfunctional conformation. Both approaches ignore the possibility that lipids modulate the equilibria between different conformational states. The relative level of a given lipid in a reconstituted phosphatidylcholine membrane may be an important factor in determining the relative percentage of nAChRs stabilized in a functional conformation and/or the kinetics of nAChR conformational change.

To gain a more conclusive picture of the specific lipid requirements of the nAChR and to test the above noted hypothesis regarding a lipid-dependent modulation of nAChR conformational equilibria, we have examined the ability of the nAChR to undergo the Carb-induced R → D conformational change in EPC membranes with varying levels of either DOPA or Chol. We find that increasing levels of either lipid in an EPC membrane increasingly stabilizes a larger proportion of nAChRs in a conformation(s) that is(are) capable of undergoing Carb-induced desensitization. However, only high levels of DOPA were found to stabilize a structure of the nAChR which is fully equivalent to that found in native and 3:1:1 EPC/DOPA/Chol membranes. These results suggest that the presence of either DOPA or Chol in a reconstituted EPC membrane can influence the equilibrium between the R and D conformational states but that anionic lipids are required for the nAChR to adopt a fully functional conformation.

**EXPERIMENTAL PROCEDURES**

**Materials**—EPC and DOPA were purchased from Avanti Polar lipids, Inc. (Alabaster, AL), and the Chol was from Sigma. Frozen Torpedo californica electric tissue was from Marinus (Long Beach, CA). The [13C]acetylecholine was synthesized from choline bromide and [13C]acetylechloride (both from Sigma) and purified according to Damle et al. (14). The infrared spectrum of the synthesized [13C]acetylecholine was superimposable on a similar spectrum of commercially available [13C]acetylecholine (15). Sample Preparation—The nAChR was affinity purified on a bromoacetylcholine bromide-derivatized Affi-Gel 102 column (Bio-Rad) and then reconstituted into membranes composed of EPC with varying levels of either DOPA or Chol, as described by McCarthy and Moore (8). In all cases except for the 9:1 molar ratios of EPC/DOPA and EPC/Chol, each reconstitution was performed between two and five times.

**FTIR Difference Spectroscopy**—FTIR samples were prepared by spreading 250 μg of the nAChR protein in 2 mM H2O buffer on the surface of a 50 × 20 × 2 mm germanium attenuated total reflectance (ATR) internal reflection element (Harrick; Ossining, NY). After evaporating the bulk solvent with a gentle stream of N2 gas, the ATR crystal was overlapped negative band near 1668 cm⁻¹. A spectrum of the D state was recorded. The difference between both the two R state spectra (absence of Carb; control spectra) and the consecutive R and D (presence of Carb) state spectra were calculated, stored, and the flowing buffer switched back to buffer without Carb. After a 20-min washing period to remove Carb from the film and convert the nAChR back into the R conformation, the process was repeated many times. Each experiment was repeated on several new films prepared from each affinity purification/reconstitution. All difference spectra were base line corrected between 1800 and 1000 cm⁻¹ and were interpolated to an effective resolution of 4 cm⁻¹.

The difference between infrared spectra of the nAChR recorded in the presence and absence of Carb (referred to as a Carb difference spectrum) exhibits a complicated pattern of positive and negative vibrational bands. These difference bands reflect changes in the vibrations of those amino acid residues in the nAChR whose structures and/or surrounding environments change upon Carb binding. The pattern of difference bands provides a spectral map of the Carb-induced structural changes that occur in the nAChR. Specifically, this map includes features indicative of: 1) the vibrations of Carb bound to the nAChR (identified by arrows in the top trace of Fig. 1A); 2) vibrational changes associated with the formation of direct physical interactions between Carb and binding site residues (e.g. hydrogen bonds, cation π-electron interactions, etc.); and 3) vibrational changes associated with the R → D conformational change. The conformational change probed in a typical Carb difference spectrum is shown schematically in Fig. 1B, scheme 1.

Carb difference spectra recorded from affinity-purified nAChR reconstituted into membranes composed of 3:1:1 EPC/DOPA/Chol, a membrane that gives rise to a strong Carb-induced cation flux (3), are similar to those recorded from the nAChR in native membranes and thus illustrate the pattern of difference bands expected for a functional nAChR (top trace in Fig. 1A). Carb difference spectra recorded from the nAChR reconstituted into EPC membranes lacking neutral and anionic lipids are similar but exhibit a number of band intensity variations (middle trace in Fig. 1A). These variations reflect subtle membrane-induced changes in the structure/conformation of the nAChR and include a marked decrease in the intensity of five positive bands centered near 1663, 1655, 1547, 1430 and 1059 cm⁻¹. The decrease in intensity of a positive band near 1663 cm⁻¹ gives rise to the apparent increase in intensity of the overlapping negative band near 1668 cm⁻¹ (see Fig. 3 and text in Ref. 16).

Although the individual bands in the Carb difference spectra remain to be assigned to specific residues, it is significant that the above noted changes in intensity are all observed in Carb difference spectra recorded from the nAChR in 3:1:1 EPC/DOPA/Chol membranes maintained in continuous contact with desensitizing local anesthetics, such as dibucaine (Fig. 1A, bottom trace). In addition, relatively low concentrations of the anesthetic tetracaine, which stabilize the nAChR in an R-like as opposed to a D conformation, lead to an increase, as opposed to a decrease, in the intensities of the same five difference bands. Both observations suggest that the five noted positive difference bands reflect changes in vibrational intensity which occur as a consequence of the R → D conformational transition itself (for a detailed discussion, see Ref. 16). The absence of these five bands in difference spectra recorded from the nAChR...
Panel A. Carb difference spectra recorded from the nAChR reconstituted into membranes composed of 3:1:1 EPC/DOPA/Chol (top trace). EPC (middle trace), and 3:1:1 EPC/DOPA/Chol but while the nAChR is maintained in the continuous presence of 200 μm dibucaine (bottom trace). The arrows in the top trace denote vibrations that result from the bound Carb. The arrows in the bottom trace denote some of the negative dibucaine vibrations that appear because of the Carb-induced displacement of dibucaine from the neurotransmitter binding site (16). The dashed lines denote some of the frequencies at which the lipid-sensitive spectral changes are observed. Panel B, schematic diagram of the conformational changes probed in a Carb difference spectrum. In a typical difference experiment, spectra are recorded in the presence and absence of Carb. The difference spectrum exhibits bands that reflect the structural changes in the nAChR which occur as a result of Carb binding and the subsequent R → D conformational transition (scheme i). If the nAChR is maintained in a D state prior to the addition of Carb by either a local anesthetic or reconstitution into a membrane composed of only EPC (see "Discussion"), the Carb difference spectrum reflects mainly the vibrational changes associated with the formation of physical interactions between Carb and the nAChR (scheme ii). In both cases vibrations due to the nAChR bound Carb are observed.

Reconstituted into EPC membranes indicates that the nAChR in this membrane environment does not undergo the R → D conformational transition upon the binding of Carb (Fig. 1B, scheme ii). This result is consistent with photoaffinity labeling studies, which show that the nAChR in EPC is stabilized in the D state (8).

In addition to the marked band intensity changes discussed above, more subtle lipid-sensitive spectral changes may occur between 1750 and 1700 cm⁻¹, 1580 and 1520 cm⁻¹, and 1400 and 1100 cm⁻¹. Changes in band intensity in the 1700–1750 cm⁻¹ region are difficult to monitor because of the overlapping Carb vibration at 1720 cm⁻¹ (Fig. 2A). To circumvent this problem, difference spectra were recorded using isotopically labeled acetylcholine ([13C] label on the carbonyl carbon) instead of Carb to induce the R → D conformational transition (Fig. 2B). The resulting [13C]acetylcholine difference spectra exhibit a clear window in the 1750–1700 cm⁻¹ region for viewing underlying protein vibrations. [13C]Acetylcholine difference spectra recorded from the nAChR in EPC/DOPA/Chol exhibit both a weak negative and positive difference band centered near 1740 and 1720 cm⁻¹, respectively, which could reflect changes in the vibrational intensity and/or frequency of either a protonated carbonyl or a lipid ester carbonyl. The 1740 cm⁻¹ vibration is absent in [13C]acetylcholine difference spectra recorded from the nAChR reconstituted into EPC membranes (Fig. 2C).

Potential variations in intensity between 1520 and 1580 cm⁻¹ and between 1400 and 1100 cm⁻¹ are difficult to assess because of the possibility of temperature-sensitive baseline fluctuations that can occur in these regions and/or the relatively weak intensities of the difference bands. Comparisons of potential spectral changes in these regions with those observed in spectra recorded in the presence of desensitizing local anesthetics are also complicated because negative local anesthetics bands due to Carb-induced displacement of local anesthetics from the neurotransmitter binding site appear in the latter spectra (Fig. 2A, bottom trace). It is thus difficult to assess both whether or not these spectral changes are present and, if present, whether the putative spectral changes are associated with the R → D conformational change. Current discussion will thus focus mainly on the six lipid-sensitive bands that are noted above near 1740, 1663, 1655, 1547, 1430, and 1059 cm⁻¹.

Increasing Levels of DOPA in EPC Membranes—Carb difference spectra recorded from the nAChR reconstituted into membranes composed of EPC with increasing molar proportions of the anionic lipid DOPA exhibit a DOPA-dependent increase in positive intensity at the five noted conformationally sensitive band frequencies centered near 1663, 1655, 1547, 1430, and 1059 cm⁻¹ (Fig. 3). In general, the increases in intensity at these five frequencies are modest at low levels of DOPA up to the EPC/DOPA molar ratio of 3:1 whereas at higher levels of DOPA they are more substantial. The intensities of all five conformationally sensitive bands in the Carb difference spectra recorded from the nAChR in EPC/DOPA at both the 3:2 and 1:1 molar ratios approach those observed in difference spectra recorded from the nAChR in 3:1:1 EPC/DOPA/Chol membranes. In addition, the [13C]acetylcholine difference spectra recorded from the nAChR in 3:2 EPC/DOPA membranes exhibit a relatively strong negative and positive band near 1740 and 1720 cm⁻¹, respectively. The difference spectra thus indicate that the nAChR in EPC/DOPA at both the 3:2 and 1:1 molar ratios recovers its ability to undergo the R → D conformational transition and thus must be stabilized predominantly in a functional R conformation. In terms of our technique, high levels of DOPA in an EPC membrane appear to be sufficient to stabilize the nAChR in a functional conformation that is equivalent to that found in 3:1:1 EPC/DOPA/Chol membranes, even in the absence of Chol.

Assuming that the nAChR in EPC membranes is stabilized in a D state (see “Discussion”), our data are consistent with a gradual shift in the equilibrium toward the R state with increasing levels of DOPA. A close examination of the data reveals that the effects of DOPA may be more complex than the modulation of a simple two-state conformational equilibrium. The difference spectra recorded from the nAChR in both the EPC/DOPA 3:1 and 9:1 membranes exhibit large positive intensities near 1655 and 1430 cm⁻¹ relative to the intensities of the bands in spectra recorded from the nAChR in EPC (see Figs. 5A and 6). In contrast, the intensities of the two vibrations near 1059 cm⁻¹ and 1663 cm⁻¹ remain weak and are similar to the intensities of the two bands in the Carb difference spectra recorded from the nAChR in EPC membranes completely lacking DOPA. This pattern of band intensity vari-
ations suggests that a large percentage of the conformationally sensitive residues in the nAChR which contribute intensity to the difference band near 1655 cm$^{-1}$ adopt an R-like conformation in 3:1 EPC/DOPA membranes, whereas the majority of the residues that contribute intensity to the difference bands near 1663 and 1059 cm$^{-1}$ adopt a D-like conformation. The EPC/DOPA 9:1 and 3:1 membranes thus appear to stabilize a conformational intermediate between the R and D states. Note that this same intermediate pattern of band intensities has been observed in Carb difference spectra recorded from the nAChR in EPC/DOPA/Chol membranes, but while the nAChR is maintained in the presence of concentrations of desensitizing local anesthetics where binding occurs exclusively to the noncompetitive blocker site located near the ion channel pore (16). A similar pattern of band intensities variations is also observed in Carb difference spectra recorded from the nAChR in both 3:1 EPC/phosphatidylserine and 3:1 EPC/squalene membranes (11).

**Increasing Levels of Chol in EPC Membranes—** Carb difference spectra recorded from the nAChR reconstituted into EPC membranes with increasing proportions of the neutral lipid Chol are similar to those recorded from the nAChR in EPC membranes with increasing levels of DOPA. There is a Chol-dependent increase in the intensities of four of the five noted conformationally sensitive bands near 1663, 1655, 1547, and 1430 cm$^{-1}$ (Fig. 4). The intensities of these four bands in the difference spectra recorded from the nAChR in 3:2 EPC/Chol approach the intensities of those recorded from the nAChR in 3:1:1 EPC/DOPA/Chol, suggesting that the nAChR has, for the most part, adopted an R-like conformation. The 3:1 EPC/Chol membranes also appear to stabilize a conformational intermediate between the R and D states as indicated by the relatively large positive intensity near 1655 cm$^{-1}$ versus the relatively weak intensity near 1663 and 1059 cm$^{-1}$ (Fig. 5B). There are, however, subtle variations between the Carb difference spectra recorded in the presence of increasing proportions of DOPA and Chol which suggest differences in the abilities of these two lipids to modulate conformational equilibria of the nAChR.

First, at equivalent levels of either DOPA or Chol in the EPC membranes, the presence of DOPA leads to a greater intensity of the conformationally sensitive bands near 1663, 1655, 1547, 1430, and 1059 cm$^{-1}$, implying that DOPA is slightly more effective at shifting the equilibrium toward the R conformation (Fig. 6). This result is in agreement with the labeling studies of McCarthy and Moore, which suggest that the nAChR in 3:1 EPC/Chol (25%) is predominantly in the D state, whereas in 3:1 EPC/DOPA it is predominantly in the R conformation (8). Second, increasing proportions of Chol have weak if any effect on the intensity of the difference band centered near 1059 cm$^{-1}$.  

**Fig. 2.** Difference spectra recorded using either Carb or $[^{13}$C]acetylcholine to induce the R$\rightarrow$D conformational transition. A small region of the difference between spectra recorded in the presence and absence of Carb from the nAChR reconstituted into 3:1:1 EPC/DOPA/Chol (top trace) is compared with the solution absorbance spectrum of Carb (bottom trace) in panel A. In panel B, the same region showing the difference between spectra recorded in the presence and absence of $[^{13}$C]acetylcholine from the nAChR reconstituted into 3:1:1 EPC/DOPA/Chol (top trace) is compared with the solution absorbance spectrum of $[^{13}$C]acetylcholine (bottom trace). Panel C compares $[^{13}$C]acetylcholine difference spectra recorded from the nAChR reconstituted into membranes composed of (from top to bottom) 3:1:1 EPC/DOPA/Chol, 3:2 EPC/DOPA, 3:2 EPC/Chol, and EPC.

**Fig. 3.** Selected regions of Carb difference spectra recorded from the nAChR in EPC membranes with increasing levels of DOPA. The molar ratio of EPC to DOPA is shown in parentheses. The top trace in each panel is the Carb difference spectrum recorded from the nAChR in 3:1:1 EPC/DOPA/Chol.

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cm\(^{-1}\).

The molar ratio of EPC to Chol is shown in parentheses. The top trace in each panel is the Carb difference spectrum recorded from the nAChR in 3:1:1 EPC/DOPA/Chol.

At all levels of Chol, the intensity near 1059 cm\(^{-1}\) remains essentially the same as the intensity of the band in difference spectra recorded from the nAChR in EPC alone. In contrast, the intensity of this band doubles in difference spectra recorded from the nAChR in either 3:1:1 EPC/DOPA/Chol, 3:2 EPC/DOPA, or 1:1 EPC/DOPA (right panel of Fig. 3). The presence of Chol also does not lead to difference spectra with negative intensity near 1740 cm\(^{-1}\) comparable to that observed in spectra recorded from the nAChR in both 3:1:1 EPC/DOPA/Chol and 3:2 EPC/DOPA membranes (Fig. 2). The lack of an effect of Chol on the intensities of these two vibrations may indicate that there are subtle structural differences between the nAChR in EPC membranes either with or without anionic lipids (see “Discussion”).

Finally, the difference spectra recorded from the nAChR reconstituted into 1:1 EPC/Chol are similar to those recorded from the nAChR in EPC/Chol 3:1 (Fig. 6). In other words, the pattern of increasing intensity at each of the conformationally sensitive difference bands near 1663, 1655, 1547, and 1430 cm\(^{-1}\) with increasing Chol is reversed at very high levels of Chol. This reversal in the pattern of difference band intensity changes suggests that the ability of Chol to stabilize the nAChR in an R-like conformation is weakened at very high levels of Chol. A similar reversal in trend is observed with the peptide \(^{1}H/\(^{2}H\) exchange kinetics of the nAChR upon reconstitution into EPC membranes with increasing levels of Chol (23).

**DISCUSSION**

The main goal of this work was to test the hypothesis that the nAChR requires neutral and anionic lipids in its surrounding membrane environment in order to adopt a functional conformation that will undergo agonist-induced conformational change. We have shown previously that the difference between FTIR spectra of the nAChR recorded in the presence and absence of Carb exhibits positive and negative bands that serve as markers of the ability of the nAChR to undergo the R→D conformational transition (11, 16). These markers are absent in Carb difference spectra recorded from the nAChR reconstituted into EPC membranes. The absence of both neutral and anionic lipids from a reconstituted EPC membrane thus leads to a receptor that cannot undergo Carb-induced conformational change. In contrast, these markers are present with increasing intensity in Carb difference spectra recorded from the nAChR reconstituted into EPC membranes with increasing levels of either DOPA or Chol. Both DOPA and Chol can thus individually influence the ability of the nAChR to undergo conformational change in response to the binding of Carb. The level of either Chol or DOPA in the reconstituted membrane, however, is a critical factor in determining the efficacy of that lipid in stabilizing a “functional” receptor.

The changes in the intensities of bands observed in Carb difference spectra recorded from the nAChR reconstituted into EPC membranes with increasing levels of either DOPA or Chol suggest several features regarding the mechanisms of lipid action at the nAChR. These features have been incorporated into the speculative model presented in Fig. 7. The basic tenet of our model is that lipid composition influences the equilibrium between the R and D states in the absence of agonist via an indirect effect on some physical property of the lipid membrane, possibly bulk membrane fluidity. We also suggest that anionic lipids, in addition to a proper membrane fluidity, are required to stabilize a functional nAChR. The model is based on the following observations and arguments.

**Lipid Composition Influences nAChR Conformational Equilibria**—The postulate that lipid composition influences nAChR conformational equilibria is based on the observation that the nAChR in EPC alone does not undergo Carb-induced conformational change, whereas in the presence of increasing levels of either DOPA or Chol, an increasing number of receptors regain the ability to undergo Carb-induced desensitization. The stabilization of a D-like state in EPC, as suggested by the
as proposed by Rankin et al. (9). We do not favor the latter interpretation for second, Rankin et al. (9) suggest that lipid composition has no effect on nAChR conformational equilibria (9). In the absence of bound ligand.

Lipids Influence Equilibria between Different nAChR Conformational States—A second postulate of our model is that lipids influence the equilibria between different conformational states of the nAChR by a nonspecific effect on some bulk property of the membrane. This postulate is based on several observations including the fact that the addition of either Chol,
DOPA, phosphatidylserine, or squalene to reconstituted EPC membrane can influence nAChR conformational equilibria (11). Mixtures of a variety of diverse neutral and anionic lipids are capable of replacing DOPA and Chol in supporting a functional nAChR (12). Chol covalently linked to a phospholipid in the bulk lipid is as effective as free Chol in stabilizing a conformationally competent nAChR, suggesting that the non-annular sites proposed by Jones and McNamee (4) are not critical (18). Only Chol interaction sites on the lipid-exposed surface of the nAChR were detected in a recent affinity labeling experiment (19). Although specific Chol binding sites on the nAChR are often invoked to explain the ability of Chol to enhance nAChR activity, both the loose structural requirements of the nAChR for neutral and anionic lipids and the lack of experimentally documented specific Chol binding sites are suggestive of an indirect effect of lipids on bulk properties of the lipid membrane.

We propose that both Chol and DOPA modulate nAChR conformational equilibria by influencing the bulk membrane fluidity as shown in Fig. 7. It is well documented that the presence of Chol in a membrane, including reconstituted membranes containing the nAChR (7), leads to an ordering of the fatty acyl chains and thus a decrease in fluidity (20). Although the effects of DOPA on the physical properties of EPC membranes are not well characterized, it is possible that the very small head group leads to a lateral condensation of the lipid acyl chains and thus an increased ordering of the lipid bilayer (21, 22). In support of the latter, it has been shown that increasing levels of DOPA in reconstituted EPC membranes lead to a decrease in the proportion of lipid ester carbonyls that are hydrogen-bonded to water, which could correspond to a decreased penetration of water into the lipid head group region of the bilayer as a result of a lateral condensation of the phospholipids (23).

Although a role for membrane fluidity in modulating nAChR function has been suggested, molecular order parameters derived from fluorescent probe studies of the nAChR in reconstituted membranes are not consistent with a correlation between bulk fluidity and function (12). However, a single order parameter describing the amplitudes of motion of a rigid fluorescent probe in a lipid bilayer may not be sufficient to describe accurately the complex motions and dynamics of the lipids themselves and thus the so-called membrane fluidity. It may be necessary to describe the rates and amplitudes for each type of lipid motion as well as other factors such as diffusion rates, lipid flip-flop times, etc. More comprehensive studies using solid state NMR are currently under way in our laboratory to assess the role of membrane fluidity in modulating the conformational equilibria of the nAChR.

nAChR Requires Anionic Lipids and Membrane Fluidity—A third postulate of our model is that the nAChR requires the specific presence of anionic lipids, in addition to the proper membrane fluidity noted above, in order to adopt a fully functional conformation. This postulate is based on the observation that Carb difference spectra recorded from the nAChR in membranes containing high levels of DOPA are essentially equivalent to those recorded from the nAChR in functional 3:1:1 EPC/DOPA/Chol and native membranes, whereas the difference spectra recorded from the nAChR in EPC membranes with high levels of Chol are not. Specifically, the Carb difference spectra recorded from the nAChR in 3:2 EPC/Chol do not exhibit the same band intensities near 1740 and 1059 cm⁻¹ which are observed in difference spectra recorded from the nAChR in either 3:1:1 EPC/DOPA/Chol, 3:2 EPC/DOPA, or 1:1 EPC/DOPA membranes. The significance of these subtle vibrational differences remains to be established but could indicate that certain residues in the nAChR are not found in a fully functional conformation in the absence of anionic lipids. Unpublished data from our laboratory also suggest that the nAChR in 3:2 EPC/DOPA interacts with local anesthetics in a manner equivalent to that observed with the nAChR in 3:1:1 EPC/DOPA/Chol, whereas in 3:2 EPC/Chol membranes significant differences in the local anesthetic-nAChR interactions are observed.³ Our preliminary evidence suggests that subtle variations in the Carb difference spectra recorded from the nAChR in EPC membranes either with or without DOPA may correlate with functional consequences for the nAChR. We tentatively refer to the R and D conformations of the nAChR stabilized in the absence of anionic lipids as pseudo-R and -D states, respectively (Fig. 7).

The observed ability of EPC/DOPA membranes to support a functional nAChR is consistent with the work of McCarthy and Moore, who showed that the nAChR in 3:1 EPC/DOPA membranes is capable of undergoing agonist-induced conformational change (8). Although usually treated as nonfunctional, the nAChR in 3:1 DOPC/DOPA does exhibit a small cation flux in response to the binding of Carb (12, 24). In contrast to our data, most recent studies have concluded that the nAChR has an absolute structural requirement for Chol. However, studies designed to test the functional state of the nAChR in the presence of anionic lipids have always assayed nAChR function in DOPC membranes with the anionic lipid of interest at a level of 25% or less of the total lipids. Only a small proportion of the nAChR in EPC membranes containing 25% or less DOPA is stabilized in a functional R state (Fig. 6), consistent with the low cation flux observed in similar membranes. It may also be that the DOPC/DOPA mixtures used by others are slightly more fluid than the EPC/DOPA mixtures used here (DOPC has two unsaturated oleoyl chains whereas EPC has predominantly one), which might lead to a greater proportion of the nAChRs in a nonfunctional state. It is interesting to note that the nAChR in DOPC/Chol mixtures does not flux cations, whereas in neutral lipid-depleted asolectin supplemented with Chol, the nAChR does flux cations (7). The latter study concluded that a component other than Chol is critical for the nAChR to adopt a functional state. Our data suggest that the high levels of Chol in both of these membranes may provide a proper fluidity. The missing component in the DOPC/Chol mixtures may be an anionic lipid, such as DOPA. The specific mechanism by which anionic lipids alone influence nAChR conformation remains to be established.

Lipids Influence Conformational Equilibria between More than a Single R and Single D State—The final postulate of our model is that lipids influence the conformational equilibria between more than a single R (or pseudo-R) and a single D (or pseudo-D) state. This postulate is based on the observation that the difference spectra recorded from the nAChR in 3:1 EPC/DOPA and 3:1 EPC/Chol membranes exhibit a pattern of bands which is suggestive of the stabilization of the nAChR in a conformation that is a structural intermediate between the R and D states. Specifically, the spectra recorded from the nAChR in both membranes exhibit intensities of the conformationally sensitive vibrations near 1663 and 1059 cm⁻¹ which are similar to those observed in difference spectra recorded from the nAChR in EPC membranes alone, whereas the intensities near 1655 and 1430 cm⁻¹ are closer to that found in difference spectra recorded from the nAChR in 3:1:1 EPC/DOPA/Chol. At these low levels of either anionic or neutral lipids, some residues of the nAChR adopt a D-like conformation, whereas others adopt an R-like conformation. Note that

³ S. E. Ryan and J. E. Baenziger, unpublished observations.
the same pattern of spectral variations is observed in Carb difference spectra recorded from the nAChR in EPC/DOPA/ Chol membranes while the nAChR is exposed to concentrations of local anesthetics which lead to exclusive binding to the noncompetitive blocker site in the ion channel pore (16). Lipids may influence the equilibria among at least three different conformational states of the nAChR.

The data presented here illustrate the potential complexity of the mechanisms by which lipids influence nAChR function. These complexities have been incorporated into the model presented in Fig. 7, which accounts for many observations in the literature regarding the effects of lipids on nAChR function. Other factors, such as the microlipid environment surrounding the nAChR may also play an important role (17). Our model presents clear hypotheses that can be tested with definitive experiments. To understand fully the mechanisms of lipid-protein interactions at the nAChR, however, comprehensive experiments of the effects of lipids on nAChR structure and function are required.

Conclusions—We have reconstituted the nAChR into EPC membranes with increasing levels of either DOPA or Chol. The increasing levels of either lipid increasingly stabilize the nAChR in a conformation that is capable of undergoing Carb-induced conformational change, although only DOPA stabilizes the nAChR in what appears to be a fully functional state. We interpret our results in terms of a speculative model that describes the mechanisms by which lipids influence nAChR function. We suggest that membrane fluidity or some other bulk property of the membrane modulates the equilibrium between the R and D states. A membrane with a relatively low fluidity may be required to stabilize the nAChR in the functional R conformation, whereas a relatively fluid membrane may lead to the stabilization of the D state. At least one conformational intermediate between the R and D states likely exists. Finally, the model suggests that in addition to an indirect effect of lipids via the bulk fluidity, the nAChR requires anionic lipids such as DOPA, but not Chol, to adopt a fully functional conformation. This specific lipid requirement for anionic lipids could result from either the binding to a specific site on the nAChR or a less specific effect of charge on nAChR conformation.

REFERENCES
1. Miyazawa, A., Fujiyoshi, Y., Stowell, M., and Unwin, N. (1999) J. Mol. Biol. 288, 765–786
2. McNamee, M. G., and Fong, T. M. (1988) in Lipid Domains and the Relationship to Membrane Function (Aloia, R. C., Curtain, C. C., and Gordon, L. M., eds) pp. 43–62, Alan R. Liss, Inc. New York
3. Fong, T. M., and McNamee, M. G. (1986) Biochemistry 25, 3871–3880
4. Jones, O. T., and McNamee, M. G. (1988) Biochemistry 27, 2364–2374
5. Butler, D. H., and McNamee, M. G. (1993) Biochim. Biophys. Acta 1150, 17–24
6. Bhushan, A., and McNamee, M. G. (1995) Biochim. Biophys. Acta 1246, 716–723
7. Fernandez-Ballester, G., Castresana, J., Fernandez, A. M., Arrondo, J. L. R., Ferragut, J. A., and Gonzalez-Bos, J. M. (1994) Biochemistry 33, 4065–4071
8. McCarthy, M. P., and Moore, M. A. (1992) J. Biol. Chem. 267, 7655–7663
9. Rankin, S. E., Addona, G. H., Kloczewiak, M. A., Bugge, B., and Miller, K. W. (1997) Biochim. Biophys. Acta 134, 2446–2455
10. Raines, D. E., and Krishnan, N. S. (1998) Biochim. Biophys. Acta 1374, 83–93
11. Ryan, S. E., Demers, C. N., Chew, J. P., and Baenziger, J. E. (1996) J. Biol. Chem. 271, 24590–24597
12. Sunshine, C., and McNamee, M. G. (1994) Biochim. Biophys. Acta 1191, 59–64
13. Méthot, N., Demers, C. N., and Baenziger, J. E. (1995) Biochemistry 34, 1542–1549
14. Damle, V. N., McLaughlin, M., and Karlin, A. (1978) Biochem. Biophys. Res. Commun. 84, 845–851
15. Williamson, P. T., Grabner, G., Spooner, P. J., Miller, K. W., and Watts, A. (1998) Biochemistry 37, 10854–10859
16. Ryan, S. E., and Baenziger, J. E. (1999) Mol. Pharmacol. 55, 348–355
17. Dreger, M., Krauss, M., Herrmann, A., and Hueco, F. (1997) Biochemistry 36, 839–847
18. Addona, G. H., Sandermann, H., Jr., Kloczewiak, M. A., Hussain, S. S., and Miller, K. W. (1998) Biochim. Biophys. Acta 1370, 299–309
19. Corbin, J., Wang, H. H., and Blanton, M. P. (1998) Biochim. Biophys. Acta 1414, 65–74
20. Yeagle, P. L. (1988) in Biology of Cholesterol (Yeagle, P. L., ed) pp. 121–146, CRC Press, Inc., Boca Raton, FL
21. Salmon, A., Dodd, S. W., Williams, G. D., Beach, J. M., and Brown, M. F. (1987) J. Am. Chem. Soc. 109, 2600–2609
22. Marcelja, S. (1974) Biochim. Biophys. Acta 334, 889–893
23. Biel, M. A., Lee, Y., and Tsien, R. Y. (1987) J. Biol. Chem. 262, 2364–2374
24. Ochoa, E. L. M., Dalziel, A. W., and McNamee, M. G. (1983) Biochim. Biophys. Acta 727, 151–162