Structural Effects of Neutral and Anionic Lipids on the Nicotinic Acetylcholine Receptor

AN INFRARED DIFFERENCE SPECTROSCOPY STUDY*

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The effects of both neutral and anionic lipids on the structure of the nicotinic acetylcholine receptor (nAChR) have been probed using infrared difference spectroscopy. The difference between infrared spectra of the nAChR recorded using the attenuated total reflectance technique in the presence and absence of the neurotransmitter analog, carbamylcholine, exhibits a complex pattern of positive and negative bands that provides a spectral map of the structural changes that occur in the nAChR upon ligand binding and subsequent desensitization. This spectral map is essentially identical in difference spectra recorded from native, native alkaline-extracted, and affinity-purified nAChR reconstituted into either soybean asolectin or egg phosphatidylcholine membranes containing both neutral and anionic lipids. This result suggests both a similar structure of the nAChR and a similar resting to desensitized conformational change in each membrane environment. In contrast, difference spectra recorded from the nAChR reconstituted into egg phosphatidylcholine membranes lacking neutral and/or anionic lipids all exhibit an essentially identical pattern of band intensity variations, which is similar to the pattern of variations observed in difference spectra recorded in the continuous presence of the desensitizing local anesthetic, dibucaine. The difference spectra suggest that the main effect of both neutral and anionic lipids in a reconstituted egg phosphatidylcholine membrane is to help stabilize the nAChR in a resting conformation. In the absence of neutral and/or anionic lipids, the nAChR is converted into an alternate conformation that appears to be analogous to the local anesthetic-induced desensitized state. Significantly, the proportion of receptors found in the resting versus the putative desensitized state appears to be dependent upon the final lipid composition of the reconstituted membrane. A lipid-dependent modulation of the equilibrium between a channel-active resting and channel-inactive desensitized state may account for the modulations of nAChR activity that are observed in different lipid membranes.

The nicotinic acetylcholine receptor (nAChR) from Torpedo is a large integral membrane protein that has been studied extensively as a model of both neurotransmitter-gated ion channel structure/function and protein-lipid interactions (1, 2). The nAChR is composed of four distinct subunits arranged as a pentamer pseudosymmetrically around a central pore that functions as a cation selective ion channel. In native membranes, roughly 80% of the nAChR exist in a resting conformation that transiently conduct cations across the membrane in response to the binding of cholinergic agonists, such as acetylcholine and carbamylcholine (Carb) (29). The remaining ~20% exist in a high affinity channel-inactive desensitized state that is stabilized by prolonged exposure to agonist and some non-competitive antagonists. The ability of the nAChR to conduct cations across the membrane and undergo the resting to desensitized affinity state transition is exquisitely sensitive to the composition of the lipid membrane that surrounds the nAChR. Previous studies have suggested that the nAChR requires an optimal membrane fluidity as well as the presence of both a neutral lipid, such as cholesterol (Chol), and an anionic lipid, such as dioleoylphosphatidic acid (DOPA), to retain optimal flux and desensitization capabilities (3–5). In the absence of Chol and DOPA, the nAChR adopts a channel-inactive conformation that may be analogous to the ligand-induced desensitized state (4).

In contrast to the well characterized effects of lipids on nAChR function, the lipid-induced changes in the structure of the nAChR that lead to modulations in function are poorly understood. The functional requirement of the nAChR for neutral and anionic lipids has been attributed to the binding of each lipid to distinct sites at or near the protein-lipid interface with distinct effects on the gross secondary structure of the nAChR (3, 8–11). Others have reported more dramatic alterations in the content of both α-helix and β-sheet, with increasing levels of Chol in reconstituted neutral lipid depleted soybean asolectin membranes (12). In contrast, a recent Fourier transform infrared (FTIR) and hydrogen/deuterium exchange study was unable to detect any definitive lipid-dependent alterations in nAChR secondary structure, suggesting that both neutral and anionic lipids modulate nAChR function through subtle, but as yet unidentified changes in nAChR structure (13).

The lack of detailed insight into the structural changes induced in the nAChR by different lipids is a consequence of the difficulties associated with the application of high resolution structure elucidating techniques to large integral membrane proteins, such as the nAChR. FTIR difference spectroscopy is one of the few techniques that can probe the structure of large integral membrane proteins at the single amino acid residue level (14). The difference technique has revealed changes in the
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orientation, protonation state, strength of hydrogen bonding, and/or environments surrounding specific Tyr, Asp, Thr, Pro, Arg, Trp, and His side chains, as well as changes in the conformation and/or orientation of the polypeptide backbone upon the absorption of light by light-activated proteins, such as bacteriorhodopsin and the photosynthetic reaction center (see Refs. 34–37 and 39, and references therein). The technique has also been adapted for probing ligand-induced conformational changes in the nAChR. FTIR spectra are repetitively recorded while alternately flowing a buffer either with or without Carb past an nAChR film deposited on the surface of a germanium internal reflection element. After extensive signal averaging, the difference between spectra of the resting and desensitized states recorded under these stringent conditions exhibits a complex, but highly reproducible, pattern of positive and negative bands that provides a “spectral map” of the structural changes that occur in the nAChR upon ligand binding and subsequent desensitization (15–17).

In this report, we have used this FTIR difference approach to examine the effects of both neutral and anionic lipids on the structure of the nAChR. Relative to the difference between spectra recorded in the presence and absence of Carb from the nAChR in native membranes, those recorded from the nAChR reconstituted into EPC membranes either with or without a variety of neutral and/or anionic lipids all exhibit subtle difference band intensity variations that reflect lipid-induced structural changes in those regions of the nAChR that are involved in ligand binding and desensitization. An essentially identical pattern of difference band intensity variations is observed upon reconstitution of the nAChR into membranes lacking either neutral, anionic, or both types of lipids. The same pattern of variations is also observed in difference spectra recorded in the continuous presence of the desensitizing local anesthetic, dibucaine (Dib). The similarity of the spectral variations suggests that neutral and anionic lipids both stabilize an equivalent resting conformation of the nAChR. In the absence of either lipid, the nAChR appears to be stabilized in a desensitized conformation, although the proportion of receptors in the desensitized state appears to be dependent upon the final lipid composition of the reconstituted membrane. A lipid-dependent modulation of the equilibrium between the channel competent resting and a putative channel-inactive desensitized state may account for the effects of a variety of different lipids on the ion flux activity of the nAChR.

EXPERIMENTAL PROCEDURES

Materials—Chol, squalene, soybean asolectin (type II-S), Carb, and Dib were purchased from Sigma. Egg lecithin (referred to as EPC), DOPA, and dioleoylphosphatidylserine (DOPS) were from Avanti Polar Lipids, Inc. (Alabaster, AL). Frozen Torpedo californica electric tissue was from Marinus (Long Beach, CA).

Sample Preparation—Native and native alkaline-extracted nAChR membranes were prepared as described previously (18), except that the relatively crude native nAChR membranes were removed from the sucrose gradients using a Pasteur pipette. The nAChR was affinity-purified on a bromoacetylcholine bromide-derivatized Bio-Rad Affi-Gel 201 column and then reconstituted into lipid vesicles composed of EPC/DOPA/Chol (molar ratio of 3:1:1), EPC/DOPA (molar ratio of 3:1), EPC/DOPS (molar ratio 3:1), EPC/squalene (molar ratio 3:1), EPC/Chol (molar ratio 3:1), or EPC as described by McCarthy and Moore (4).

FTIR samples were prepared by spreading 250 µg of the nAChR protein on the surface of a 50 mm × 20 mm × 2 mm germanium attenuated total reflectance (ATR) crystal (Harrick, Ossining, NY). After evaporating the bulk solvent with a gentle stream of N2 gas, the ATR crystal was installed in an ATR liquid sample cell (also from Harrick) and the nAChR film rehydrated with excess Torpedo Ringer buffer (250 mM NaCl, 5 mM KCl, 2 mM MgCl2, 3 mM CaCl2, and 5 mM Na2HPO4, pH 7.0). Note that each nAChR film is spread over an equivalent surface area on the ATR crystal, and each expands beyond the effective penetration depth of the infrared beam upon hydration. The absolute intensities of the protein bands in the FTIR spectra are similar from sample to sample.

FTIR Spectroscopy—FTIR spectra were acquired using the ATR technique on a PTV-40 spectrometer equipped with a DTGS detector. Spectra were recorded at 8 cm−1 resolution using 512 scans, which took roughly 7 min/spectrum. For the difference measurements, two consecutive spectra of the same nAChR film in the absence of Carb were recorded with buffer flowing continuously through the sample compartment of the ATR cell at a rate of ~1.5 ml/min. The flowing solution was then switched to an identical one with 50 µM Carb. After 1 min, a spectrum was recorded of the desensitized state. The difference between both the two resting state spectra (absence of Carb; control spectra) and the consecutive resting and desensitized (presence of Carb) state spectra were calculated, stored, and the flowing buffer switched back to buffer without Carb. After a 23-min washing period to remove Carb from the film and convert the nAChR back into a resting conformation, the process was repeated many times. Each experiment was repeated on several new films prepared from at least two separate affinity purification/reconstitutions of the nAChR. The 50 µM Carb solution is sufficient to desensitize the nAChR (15), but low enough to allow the rapid washing of Carb from the nAChR film. All difference spectra were base-line corrected between 1800 and 1000 cm−1 and were interpolated to an effective resolution of 4 cm−1.

RESULTS

FTIR spectra of a thin film of the nAChR deposited on the surface of a germanium ATR crystal were recorded while alternately flowing buffer either with or without Carb past the film surface. After extensive signal averaging, the difference between spectra recorded under these stringent conditions in the presence and absence of Carb exhibits a highly reproducible pattern of positive and negative bands (Fig. 1, A and B, top three traces) that is absent in control difference spectra recorded from nAChR films preincubated with the essentially irreversible competitive antagonist, α-bungarotoxin (17). Parallel fluorescence experiments demonstrate that non α-bungarotoxin-treated nAChR films retain the ability to bind agonists.
non-competitive antagonists and undergo the resting to desensitized affinity state transition (15, 16). Bands in the difference spectra therefore reflect the molecular vibrations of those amino acid residues whose structures change upon ligand binding and desensitization. In general, negative bands reflect the vibrations of functional groups in the absence of Carb (resting state). Positive bands reflect the vibrations of the same functional groups in the presence of Carb (desensitized state), as well as those of the nAChR-bound Carb itself (17).

The absolute intensities of bands in the difference spectra recorded from native (Fig. 1A, trace a), native alkaline-extracted (Fig. 1A, trace b), and affinity-purified nAChR reconstituted into EPC/DOPA/Chol (Fig. 1A, trace c) increase with increasing purity of the nAChR preparations providing additional support for the specificity of the bands to Carb-induced structural changes in the nAChR. Expansion of the y axis shows that the frequencies and relative intensities of the majority of the positive and negative bands are similar in all three difference spectra, although subtle variations are clearly evident in the bands near 1668 and 1544 cm⁻¹ (for example compare the relative intensities of the two bands near 1544 and 1516 cm⁻¹ in each spectrum) and may exist near 1744, 1655, 1430, and 1059 cm⁻¹ (Fig. 1B). The reproducibility of the majority of the bands in the difference spectra suggests that the structures and environments surrounding the majority of those residues involved in ligand binding and/or the resting to desensitized conformational change are essentially identical in native, native alkaline-extracted, and reconstituted EPC/DOPA/Chol membranes. The slight variations in the relative intensities of the noted difference bands, which are difficult to assess due to the especially weak intensities of bands in the difference spectra recorded from native membranes, may suggest a slight increase in the proportion of the receptors found in the desensitized state in EPC/DOPA/Chol relative to the proportion of desensitized receptors found in native membranes (see below). Note that the 4- and 3-fold expansion of the difference spectra in traces a and b (panel B), respectively, emphasizes the base-line distortions that are particularly evident in the 1600 cm⁻¹ to 1700 cm⁻¹ region. These broad “rolling” base-line distortions that affect the apparent relative intensities of some of the relatively sharp difference bands are mainly due to slight instabilities in temperature over the time course of the experiment that lead to changes in the shape of the intense, broad, overlapping water absorbance near 1640 cm⁻¹.

The frequencies and relative intensities of the majority of the bands in the difference spectra recorded from the nAChR reconstituted into membranes composed of either EPC/DOPA, EPC/Chol, or simply EPC are also similar to those observed in difference spectra recorded from the nAChR in both native and EPC/DOPA/Chol membranes, suggesting that neither Chol nor DOPA dramatically affect the structures and/or environments surrounding a large number of residues involved in both ligand binding and desensitization (Fig. 2). However, there are substantial lipid-dependent variations in the intensities of bands near 1744, 1668, 1655, 1544, 1430, and 1059 cm⁻¹ that reflect lipid-induced changes in the structure of the nAChR that are likely responsible for modulations in nAChR function. The lipid-sensitive bands near 1668, 1655, and 1544 cm⁻¹ occur in either the amide I (1600–1700 cm⁻¹) or amide II (1520–1580 cm⁻¹) regions of the infrared spectrum and likely reflect a subtle lipid-induced change in the conformation of the polypeptide backbone. The assignment of these three bands to either amide I or amide II vibrations is consistent with the downshifts in the frequencies of the bands upon exposure of the nAChR to

![Image](Image345x577 to 517x729)

**Fig. 2.** The difference between FTIR spectra recorded in the presence and absence of 50 μM Carb from affinity-purified nAChR reconstituted into membranes composed of EPC/DOPA/Chol at a molar ratio of 3:1:1 (top trace), EPC/DOPA at a molar ratio of 3:1 (second from top trace), EPC/Chol at a molar ratio of 3:1 (second from bottom trace), and EPC (bottom trace). The presented difference spectra are the average of 57, 38, 37, and 26 spectral differences, respectively.

$^2$H$_2$O (33). Other lipid-sensitive bands near 1744, 1430, and 1059 cm⁻¹ could reflect either a lipid-induced change in the structure and/or environment surrounding amino acid side chains or possibly an alteration in the interactions between the nAChR and a “bound” phospholipid (see “Discussion”).

Although a detailed interpretation of the individual band intensity variations is not yet possible, it is noteworthy that the absence of Chol, DOPA, or both lipids leads to a decrease in intensity near 1744, 1655, 1544, 1430, and 1059 cm⁻¹ accompanied by an increase in negative intensity near 1668 cm⁻¹. The striking reproducibility of the pattern of band intensity variations suggests that the absence (and conversely the presence) of either or both lipids lead to a similar change in the structures of those residues of the nAChR that are involved in ligand binding and desensitization. The reproducibility of the spectral variations suggests that both Chol and DOPA stabilize an equivalent conformational state of the nAChR.

In contrast, the relative magnitudes of the intensity variations and thus the degree of the conformational perturbation appears to be dependent upon the final lipid composition of the reconstituted membrane. Relative to native and EPC/DOPA/Chol membranes, the variations in intensity of all six bands are minimal for the nAChR in EPC/DOPA, slightly more substantial for the nAChR in EPC/Chol, and relatively dramatic for the nAChR in EPC, where both the negative and positive bands near 1744 and 1655 cm⁻¹ are completely absent, the two bands near 1544 cm⁻¹ and 1430 cm⁻¹ are both reduced in intensity by more than 50%, and the negative band near 1668 cm⁻¹ is enhanced in intensity by ~100%. The relative magnitudes of the lipid-induced changes in intensity of the four most intense bands near 1668, 1655, 1544, and 1430 cm⁻¹ are summarized in Table I. The differing magnitudes of the variations in intensity of all six bands suggest that the structure of the nAChR is increasingly perturbed from a native like (predominantly resting) conformation in EPC/DOPA/Chol to an alternate, possibly desensitized conformation (see below) in EPC/DOPA, EPC/Chol, and EPC. Chol and DOPA may therefore differ in their relative abilities to stabilize the nAChR in a channel-competent resting conformation as opposed to their ability to stabilize the nAChR in distinct conformational states.

The possibility that diverse lipid structures stabilize an equivalent conformation of the nAChR was examined further by recording difference spectra from the nAChR reconstituted into soybean asolectin, EPC/DOPS, and EPC/squalene (Fig. 3).
were all normalized with respect to the area of the Carb vibration near 1668 cm\(^{-1}\), the intensities of the three bands near 1668, 1655, and 1430 cm\(^{-1}\) could not accurately be determined in the difference spectra recorded from the nAChR in native and native alkaline-extracted membranes.

### TABLE I

| Membrane composition | 1668 cm\(^{-1}\) | 1655 cm\(^{-1}\) | 1544 cm\(^{-1}\) | 1430 cm\(^{-1}\) |
|----------------------|-----------------|-----------------|-----------------|-----------------|
| Native               | ND              | ND              | ND              | ND              |
| Alkaline-extracted   | ND              | ND              | ND              | ND              |
| Asolectin            | 100             | 100             | 100             | 100             |
| EPC/DOPA/Chol        | 160             | 100             | 90              | 100             |
| EPC/DOPA             | 250             | 60              | 50              | 80              |
| EPC/squalene         | 270             | 60              | 40              | 80              |
| EPC/DOPS             | 270             | 50              | 30              | 70              |
| EPC/Chol             | 280             | 50              | 20              | 80              |
| EPC                  | 320             | 30              | 20              | 50              |

\(^{a}\) ND, not determined.

Soybean asolectin is a complex mixture of neutral, anionic, and zwitterionic lipids. The difference spectra recorded from the nAChR reconstituted into asolectin membranes are similar to those recorded from the nAChR in both native and EPC/DOPA/Chol membranes, particularly in the intensities of the six lipid-sensitive bands noted above, suggesting a similar structure of the nAChR and resting to desensitized conformational change in complex membranes containing neutral, anionic, and zwitterionic lipids.

DOPS is a negatively charged lipid with a much larger head group than DOPA, whereas squalene is an isoprenoid polynene (neutral lipid) with little structural resemblance to DOPA, DOPS, or Chol. In agreement with the previous data, the difference spectra recorded from the nAChR in both EPC/squalene and EPC/DOPS are similar to those recorded from the nAChR in native and EPC/DOPA/Chol membranes but exhibit variations in intensity of the same six bands noted above near 1744, 1668, 1655, 1544, 1430, and 1059 cm\(^{-1}\) (Fig. 3). In addition, the magnitudes of the variations in intensity of these six bands are less in EPC/squalene than in EPC/DOPS, suggesting that squalene is slightly more efficient than DOPS at stabilizing the nAChR in a native-like resting conformation (Table I). The similarity of the pattern of band intensity variations observed in difference spectra recorded from the nAChR in EPC/Chol, EPC/DOPA, EPC/DOPS, and EPC/squalene relative to the difference spectra recorded from the nAChR in EPC membranes lacking both neutral and anionic lipids strongly suggests that Chol, DOPA, DOPS, and squalene all stabilize an equivalent conformation of the nAChR.

Some evidence indicates that the nAChR is stabilized in a desensitized conformation in EPC membranes lacking both neutral and anionic lipids. This possibility implies that the difference between spectra recorded in the presence and absence of Carb from the nAChR in EPC reflect changes in the structure and/or environments surrounding residues of the nAChR upon the binding of Carb to a predominantly desensitized population of receptors. To test this possibility, the difference between spectra of the nAChR in EPC/DOPA/Chol recorded in the presence and absence of Carb were monitored while continuously maintaining the nAChR in contact with increasing concentrations of the desensitizing local anesthetic, Dib (i.e. Dib is included in both the +Carb and −Carb buffers). Increasing concentrations of Dib lead to dose-dependent variations in the intensities of a number of bands including the same six bands near 1744, 1668, 1655, 1544, 1430, and 1059 cm\(^{-1}\) that are sensitive to the absence of neutral and anionic lipids from the reconstituted membrane (Fig. 4). The striking similarity of the subtle variations in the difference spectra (other variations are discussed below) suggest that both the presence of Dib and the reconstitution of the nAChR into EPC membranes lacking neutral and/or anionic lipids lead to the stabilization of the same desensitized conformation (Fig. 5).

Note that the Dib-induced variations in the difference spectra saturate at concentrations of ~200 μM, consistent with the binding of Dib to the ion channel pore of the nAChR in native and reconstituted EPC/DOPA/Chol membranes with K_D values, in the absence of Carb, of roughly 80 μM and 30 μM, respectively (21, 40). The difference spectra also exhibit a dose-dependent increase in negative band intensity at frequencies that correspond to the vibrational frequencies observed in the solution spectrum of Dib (Fig. 5). The latter is consistent with the additional binding of Dib to the neurotransmitter binding
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ATR technique from a 250 mM aqueous solution of Dib. The overlapping bands in the difference spectra reflect structural changes that undergo the resting to desensitized affinity state transition or fluorescence spectroscopy demonstrates that similar nAChR films undergo the resting to desensitized affinity state transition under parallel experimental conditions, suggesting that the bands in the difference spectra reflect structural changes that occur upon the binding of Carb and subsequent desensitization (15).

We show here for the first time that this spectral map of the structural changes that occur upon ligand binding and desensitization is sensitive to the composition of the lipid membrane that surrounds the nAChR as well as to the presence of a local anesthetic, Dib. The lipid- and anesthetic-induced variations in the difference spectra suggest subtle changes in the conformation and/or orientation of the polypeptide backbone as well as perturbations in the structure of individual amino acid side chains and/or possible modulations of the interactions between the nAChR and a bound phospholipid (see below). Significantly, the pattern of the lipid-induced variations in the difference spectra correlate with the pattern of the effects of the lipids on both the ion flux properties and the conformational state of the nAChR, as probed using biochemical techniques (this correlation is discussed below). In addition, the effects of Dib are dose-dependent and saturate at concentrations consistent with the measured $K_D$ values for the binding of Dib to both the ion channel pore and the neurotransmitter binding site. The close correlation between the spectroscopic and biochemical data provides further confidence in the difference technique and suggests that the lipid-induced variations detected in the difference spectra are reflecting changes in structure of the nAChR that are related to lipid-dependent modulations of nAChR activity. The data also illustrate the utility of the FTIR difference technique for probing the structural changes induced in the nAChR by a variety of functional modulators and suggest three important features regarding the mechanisms of lipid action at the nAChR.

(i) The most striking feature of the data is that the difference spectra recorded from the nAChR in diverse reconstituted membranes all exhibit an essentially identical pattern of band intensity variations relative to the difference spectra recorded from the nAChR in native membranes. In particular, the reproducibility of the pattern of variations induced in the difference spectra by the inclusion of either a neutral lipid, such as Chol or squalene, or an anionic lipid, such as DOPA or DOPS, in an EPC membrane relative to the difference spectra recorded from the nAChR in EPC membranes lacking both neutral and anionic lipids suggests that Chol, squalene, DOPA, and DOPS all have an essentially identical effect on the structures and environments surrounding those residues of the nAChR that are involved in ligand binding and desensitization. Given the previously demonstrated sensitivity of the difference technique to subtle changes in protein structure, the reproducibility of these spectral variations implies that Chol, squalene, DOPA, and DOPS each stabilize an essentially identical conformational state of the nAChR.

(ii) The similarity of the pattern of band intensity variations observed in difference spectra recorded from the nAChR reconstituted into EPC membranes lacking neutral and/or anionic lipids and from the nAChR in EPC/DOPA/Chol, but in the continuous presence of increasing concentrations of Dib, suggests that both the absence of neutral and anionic lipids and the presence of Dib lead to the stabilization of the same conformational state. Dib is a member of a class of local anesthetics that bind near the ion channel pore and stabilize the desensitized conformation of the nAChR in native membranes (19, 20). Although the conformation of the nAChR stabilized by Dib in EPC/DOPA/Chol has not been defined, the nAChR in EPC/DOPA/Chol retains the ability to conduct cations (3, 6, 7) and undergoes an essentially identical resting to desensitized conformational change, as judged by the similarity of the difference spectra recorded from the nAChR in native and EPC/DOPA/Chol membranes as well as other biochemical assays (4,
The stabilization of a desensitized nAChR in EPC membranes lacking both neutral and anionic lipids is consistent with both the labeling pattern of the nAChR in EPC by the conformational sensitive probe, $^{125}$I-labeled 3-trifluoromethyl-3-($m$-$^{125}$I)iodophenyl)diazirine, and the inability of the nAChR to either conduct cations across the membrane or undergo the resting to desensitized affinity state transition (3, 4). A gradual increase in the proportion of receptors in a channel-inactive desensitized conformation in membranes with varying lipid compositions is also consistent with both the labeling pattern of the nAChR in EPC by the conformational sensitive probe, $^{125}$I-labeled 3-trifluoromethyl-3-($m$-$^{125}$I)iodophenyl)diazirine. The inability of the nAChR to undergo ligand-induced conformational transitions is enhanced in EPC/DOPA (3, 6, 7). This effect may intercalate into the grooves of α-helices stabilizing trans-membrane α-helical secondary structures, whereas the negatively charged head group of anionic lipids may form electrostatic interactions with extra membranous regions of the nAChR. Changes in the content of either α-helix or β-sheet should lead to changes in the structures and environments of a large number of residues, especially in the case of a compact, highly cooperative protein such as the nAChR. Changes in the structure and environments surrounding a large number of side chains should lead to variations in the frequencies and relative intensities of a large number of bands in the difference spectra, which is certainly not the case. Finally, a recent FTIR and hydrogen/deuterium exchange study was unable to detect any definitive lipid-dependent alterations in nAChR secondary structure and attributed the lipid-induced changes in secondary structure reported by others to either a previously unappreciated dependence of the shape of the secondary structure sensitive amide I band to the time of exposure of the nAChR to $^{2}$H$_{2}$O, and thus the extent of peptide $^{1}$H/$^{2}$H exchange, or to errors resulting from uncompensated water vapor (13). The effects of water vapor on resolution enhanced FTIR spectra of the nAChR are discussed in detail in Ref. 22 (see also Ref. 24). Lipid-dependent modulation of nAChR function must occur via subtle changes in structure of the nAChR as opposed to the stabilization of α-helix and/or β-sheet.
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zation of α-helix and β-sheet, respectively, and alter nAChR function via similar changes in structure, then alternate mechanisms are required to explain the apparent functional requirement of the nAChR for both neutral and anionic lipids. One possibility is that diverse lipid structures influence nAChR structure (i.e. the equilibrium between the resting and desensitized states) via nonspecific alterations of the physical properties of the lipid membrane. Chol has a well characterized ordering effect on lipid bilayers in the liquid-crystalline state. The very small head group of DOPA should lead to a condensation and thus a restriction in the amplitudes of motion of the fatty acyl chains and thus a more ordered, less fluid lipid bilayer (26, 27). The presence of both lipids could lead to a relatively ordered lipid membrane that might be required for maintaining the nAChR in a predominantly resting conformation. Conversely, a more fluid system in the absence of either or both lipids might increasingly enhance the formation of the desensitized conformation. This possibility could account for both the reduced ion flux capabilities and the more substantial band intensity variations detected in the difference spectra recorded from the nAChR in EPC/DOPS relative to the nAChR in EPC/DOPA (3, 7). DOPS has a large head group that would not influence the physical properties of the lipid bilayer in the same manner as DOPA. A recent spin-labeled study has also shown that the negative charge on DOPA is not responsible for specific interactions at the nAChR-lipid interface (27). Furthermore, the nAChR is stabilized in a high affinity conformation (likely desensitized) in the presence of several detergents, which disrupt the integrity and thus modulate the fluidity of the lipid membrane (4).

A potential role for membrane “fluidity” in modulating nAChR function has been suggested previously, although modulations in fluidity, as monitored using either spin-labeled fatty acid or fluorescent probes, cannot completely explain lipid-dependent modulation of nAChR activity (3, 5). However, the lack of a clear correlation between membrane fluidity and nAChR function could reflect an inability to accurately characterize the complex structure and dynamics of a lipid bilayer by monitoring the motions of either a rigid fluorescent or a spin-labeled probe. Spectroscopic methods are generally sensitive to motions occurring within a limited time frame (28, 30) and may not provide a comprehensive description of the complex variations in membrane structure and dynamics brought about by changes in membrane lipid composition. More comprehensive analyses of the effects of different lipids on membrane structure and dynamics, such as those that can be performed using a combination of NMR spectroscopy and molecular modeling (30), may be required in order to fully understand the role of membrane fluidity in the modulations of nAChR structure/function.

A second possibility is that both neutral and anionic lipids bind to distinct sites on the nAChR, but that the main effect of binding is a modulation of the equilibrium between the resting and desensitized states, as opposed to the stabilization of specific secondary structures. This possibility is supported by the observed binding of Chol to non-anular sites of the nAChR (38). The lipid-sensitive band in the difference spectrum near 1744 cm⁻¹ could also reflect a change in a phospholipid C=O stretching vibration upon the binding of Carb to the nAChR and subsequent desensitization. Preliminary studies suggest that the band does not downshift in frequency in difference spectra recorded in H₂O buffer, consistent with the assignment of the 1744 cm⁻¹ band to a lipid ester carbonyl. A possible change in the structure of a lipid upon the binding of Carb to the nAChR would imply a tight association between the phospholipid and the receptor. However, the lipid-induced changes in intensity of the weak band near 1744 cm⁻¹ must be interpreted with caution, given the sensitivity of the apparent intensity of the band to baseline fluctuations (see Fig. 1, trace a).

Future studies using isotopically labeled agonists and lipids should lead to a definitive interpretation of the lipid-sensitive bands in the 1700–1750 cm⁻¹ region that may reflect the binding of phospholipids to distinct sites on the nAChR. We are also studying the effects of local anesthetics on reconstituted nAChR membranes in order to define the conformation that is stabilized by both Dib and reconstitution into neutral and anionic lipid-depleted membranes. The possibility that the difference spectra recorded from the nAChR in either EPC or EPC/DOPA/Chol plus Dib reflect the binding of Carb to an already desensitized receptor suggests that the bands near 1744, 1668, 1544, 1435, and 1059 cm⁻¹ are reflecting the resting to desensitized conformational change itself, whereas other bands in the difference spectra reflect structural changes at or near the ligand binding that occur as a result of specific Carb-nAChR interactions. Further analyses of these and other vibrations should lead to a better understanding of both the nature of the structural changes that occur upon desensitization and the structural basis for nAChR-agonist interactions.

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