Structure of Both the Ligand- and Lipid-Dependent Channel-Inactive States of the Nicotinic Acetylcholine Receptor Probed by FTIR Spectroscopy and Hydrogen Exchange†

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ABSTRACT: FTIR spectra have been recorded both as a function of time and after prolonged exposure to ^2H_2O buffer in order to study the structural changes that lead to both the ligand- and lipid-dependent channel-inactive states of the nicotinic acetylcholine receptor (nAChR). The hydrogen/deuterium exchange spectra provide insight into both the overall rates and extent of peptide $^1H/^2H$ exchange and the individual rates and extent to which peptide hydrogens in α-helix and β-sheet conformations exchange for deuterium. The spectra are also sensitive to the conformation of the polypeptide backbone and thus the secondary structure of the nAChR. The various spectral features monitored in the presence and absence of carbamylcholine and tetracaine are essentially identical, indicating that there are no large net changes in secondary structure in the channel-inactive desensitized state. The various spectral features monitored for the nAChR reconstituted into lipid membranes either with or without cholesterol are very similar, indicating that cholesterol is not a major structural regulator of the nAChR. However, in the absence of both cholesterol and anionic lipids, there is a slightly enhanced rate of exchange of α-helical peptide hydrogens for deuterium that occurs as a result of either an increase in nAChR dynamics or an increase in the accessibility of transmembrane peptide hydrogens to ^2H_2O. The latter may simply be due to an increase in the "fluidity" and thus permeability of the lipid bilayers to aqueous solvent. The results indicate that channel inactivation is due to a very subtle change in structure of the nAChR, regardless of whether the inactive state is stabilized by either prolonged exposure to carbamylcholine or reconstitution into lipid membranes lacking cholesterol and anionic lipids. The data also illustrate the sensitivity of the amide I band shape to peptide $^1H/^2H$ exchange. Sample variations in the extent of peptide $^1H/^2H$ exchange can lead to changes in the amide I band that are easily misinterpreted in terms of a change in protein secondary structure.

The nicotinic acetylcholine receptor (nAChR)[1] from the electric organ of *Torpedo* is a large integral membrane protein (~290 000 daltons) that serves as a model for probing ligand-gated ion channel structure and function. In native membranes, the nAChR responds to the binding of cholinergic agonists, such as acetylcholine and carbamylcholine (Carb), by transiently opening a cation-selective ion channel across the postsynaptic membrane [for recent reviews, see Galzi et al. (1991) and Pradier and McNamee (1992)]. Prolonged exposure to agonist and some noncompetitive antagonists leads to the formation of a high-affinity channel-inactive or desensitized state. Both the ligand binding site and ion translocating domain are part of the nAChR pentamer ($\alpha_2\beta\gamma\delta$), which can be affinity-purified and reconstituted into synthetic lipid membranes. Upon reconstitution into lipid membranes containing cholesterol and anionic lipids, the nAChR retains the ability to bind ligands, conduct cations across the membrane, and undergo the resting to desensitized affinity-state transition (Fong & McNamee, 1986; McCarthy & Moore, 1992; Méthot et al., 1994). In lipid membranes without cholesterol and/or anionic lipids, the nAChR adopts a channel-inactive conformation that may be analogous to the ligand-induced desensitized state [see McCarthy and Moore (1992) and references cited within].

In the absence of high-resolution structural information, the structural changes responsible for both ligand- and lipid-dependent channel inactivation have been studied using physical techniques, such as Fourier transform infrared (FTIR) spectroscopy. Most FTIR spectra recorded in the presence or absence of Carb are essentially identical, suggesting that the global secondary structure is unaffected and that desensitization results from a subtle change in structure of the nAChR (Butler & McNamee, 1993; Méthot et al., 1994). The lack of a large change in secondary structure in the desensitized state is supported by CD (Mielke & Wallace, 1988; Wu et al., 1990), hydrogen−tritium exchange (McCarthy & Stroud, 1989), and Raman spectroscopy (Aslanian et al., 1993). The Carb-induced change in the peptide backbone detected by FTIR difference spectroscopy is restricted to relatively few amino acid residues (Baenziger et al., 1993).

In contrast, both Castresana et al. (1992) and Fernandez-Ballester et al. (1994) have interpreted Carb-induced changes in FTIR spectra of the nAChR in terms of a decrease in the β-sheet content from 49% down to 24% with a corresponding

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; FTIR, Fourier transform infrared; CD, circular dichroism; Carb, carbamylcholine; Tet, tetracaine; DOPC, dioleoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid; ATR, attenuated total reflectance; TID, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine.

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increase in unordered loop structures (Castresana et al., 1992). Substantial changes in structure have also been reported for the channel-inactive conformation(s) that is (are) stabilized upon reconstitution into lipid membranes lacking cholesterol and/or anionic lipids (Fong & McNamee, 1987; Butler & McNamee, 1993; Fernandez-Ballester et al., 1994) and that may be analogous to the ligand-induced desensitized state (McCarthy & Moore, 1992). McNamee and co-workers interpreted changes in FTIR spectra of the nAChR recorded in the absence of cholesterol in terms of a possible change in the α-helical character of each of the pore-lining transmembrane segments and suggested that the altered secondary structure may lead to the inactivation of the ion channel pore (Fong & McNamee, 1987; Butler & McNamee, 1993). Fernandez-Ballester et al. (1994) attributed changes in FTIR spectra recorded in the absence of cholesterol (membranes composed of neutral lipid-depleted asolectin) to a dramatic destabilization of both α-helix and β-sheet secondary structures, which decreased from roughly 45% and 50%, respectively, in the presence of 40 mol % cholesterol down to near 30% and 40%, respectively, in the absence of cholesterol.

In order to clarify the nature of the structural changes associated with the various channel-inactive conformations, we have recorded FTIR spectra of the nAChR both as a function of time and after prolonged exposure to ^2H_2O buffer in the presence and absence of two ligands, Carb and tetracaine (Tet), and for the nAChR reconstituted into lipid membranes either with or without cholesterol and/or anionic lipids. The FTIR spectra recorded as a function of time after exposure to ^2H_2O provide insight into both the overall rates of peptide $^1H/^2H$ exchange as well as the individual rates of exchange of α-helix and β-sheet peptide hydrogens (Baenziger & Méthot, 1995). Spectra recorded after prolonged exposure to ^2H_2O provide insight into the extent of peptide $^1H/^2H$ exchange and are sensitive to the conformation of the polypeptide backbone and thus the secondary structure of the nAChR. A detailed analysis of the various spectral features clearly demonstrates that the secondary structure of the nAChR is essentially unaffected by channel inactivation, regardless of whether the inactive state is stabilized by either prolonged exposure to Carb or reconstitution into lipid membranes lacking cholesterol and anionic lipids. However, when the nAChR is reconstituted into lipid membranes lacking both cholesterol and anionic lipids, there is an increase in the rates of peptide $^1H/^2H$ exchange that leads to spectral changes in the amide I band that are similar to the spectral changes interpreted by others in terms of a lipid-dependent conformational change. The results indicate that sample variations in the extent of peptide $^1H/^2H$ exchange must be carefully monitored in order to accurately interpret variations in the amide I band in terms of a change in protein secondary structure.

MATERIALS AND METHODS

Materials. Cholesterol, Carb, and tetracaine (Tet) were purchased from Sigma (St. Louis, MO). Egg lecithin (referred to as DOPC) and dioleoylphosphatidic acid (DOPA) were from Avanti Polar Lipids, Inc. (Alabaster, AL). Deuterium oxide was from Aldrich (Milwaukee, WI). Frozen *Torpedo californica* electric tissue (Marinus; Long Beach, CA) was transported to the laboratory in dry ice and stored at −80 °C.

Sample Preparation. The nAChR was affinity-purified on a bromoacetylcholine bromide derivatized Bio-Rad Affi-Gel 201 column (Richmond, CA) and then reconstituted into lipid vesicles composed of either DOPC/DOPA/cholesterol (molar ratio of 3:1:1), DOPC/DOPA (molar ratio of 3:1), or DOPC as described in detail in McCarthy and Moore (1992). During the affinity purification, the nAChR was washed extensively with high concentrations of lipids [see McCarthy and Moore (1992)] to ensure complete exchange of all native lipids. In DOPC/DOPA/cholesterol, the nAChR is functional and retains the ability to undergo channel gating and ligand-induced desensitization (Fong & McNamee, 1986; McCarthy & Moore, 1992; Méthot et al., 1994). Samples were stored at −80 °C as 100 μL aliquots in phosphate buffer, pH 7.0, containing 250 μg of nAChR protein.

The FTIR samples were prepared by spreading one aliquot of the reconstituted nAChR membranes 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 N_2 gas, the ATR crystal was immediately 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 $MgCl_2$, 3 mM $CaCl_2$, and 5 mM Na_2HPO_4, pH 7.0). Previous studies have shown that nAChR samples prepared in this manner retain the ability to reversibly bind agonists and noncompetitive antagonists and to undergo the resting-to-desensitized state transition (Baenziger et al., 1992a,b).

FTIR Spectroscopy. FTIR spectra were acquired using the ATR technique on an FTS-40 spectrometer equipped with both an MCT and a DTGS detector. The spectrometer was purged with dry air (dew point −100 °C) from an air dryer (Balston; Haverhill, MA). Spectra for deconvolution were recorded using the MCT detector at 2 cm^{-1} resolution and represent the average of 16 000 scans. The large number of scans ensures a high signal-to-noise ratio for resolution enhancement. Spectral deconvolution was performed using GRAMS (Galactic Industries; Salem, NH) and $\gamma = 4$ and a smoothing factor of 0.25. Prior to deconvolution, all spectra were stringently examined for the presence of water vapor as described in detail in Reid et al. (1995). Briefly, the spectra were deconvolved using a $\gamma = 1.5$ and a smoothing of 1.0, and were compared to water vapor spectra deconvolved using the same parameters. Any residual water vapor bands were subtracted from the spectra in order to minimize their effect on the secondary structure analysis.

The hydrogen exchange experiments were performed by first equilibrating an nAChR film on the ATR crystal with ^1H_2O. After the acquisition of one spectrum, the ^1H_2O buffer was removed from the sample compartment and replaced with ^2H_2O buffer either with or without 1 mM carbamylcholine or 5 mM tetracaine. Several spectra were recorded at 2 cm^{-1} resolution over the next 12 h with increasing numbers of scans varying from 50 to 1500 scans per spectrum (DTGS detector). All experiments were performed at 22.5 °C.

RESULTS

FTIR spectra recorded as a function of time after exposure of the nAChR to ^2H_2O buffer reveal a number of spectral changes that reflect the exchange of peptide N−^{1}H for N−^{2}H

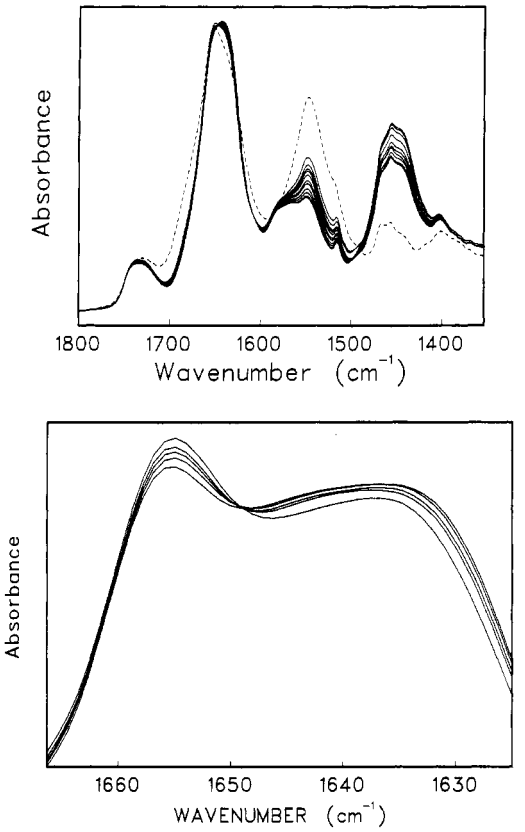

FIGURE 1: (Top panel) FTIR spectra recorded as a function of time after exposure of the nAChR reconstituted into DOPC/DOPA/cholesterol to ^2H_2O. The spectra were recorded at 2 cm^{-1} resolution after 3, 6, 9, 12, 22, 50, 80, 170, 360, 600, and 780 min of ^2H_2O exposure (solid lines from top to bottom at 1547 cm^{-1}). The dashed line spectrum was recorded in ^1H_2O. The bands due to the vibrations of both ^1H_2O and ^2H_2O have been removed by spectral subtraction. Spectra are not normalized. (Bottom panel) The resolution-enhanced amide I band in spectra recorded during the 1st, 2nd, 3rd, 6th, and 12th h after exposure of the nAChR reconstituted into DOPC/DOPA/cholesterol to ^2H_2O, in the absence of ligand. All the resolution-enhanced spectra are plotted over the same *Y* limits.

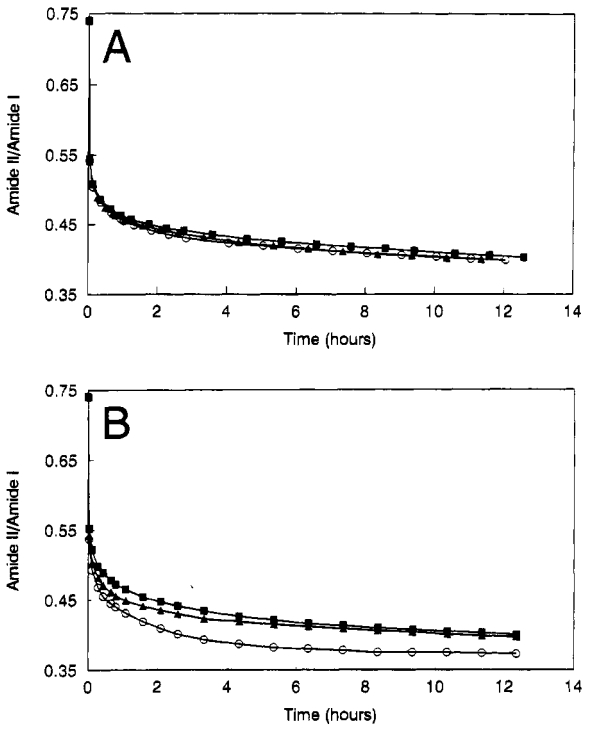

FIGURE 2: Time course of peptide N−^{1}H/N−^{2}H exchange plotted as a function of the amide II/amide I ratio. (A) Data were taken from spectra of the nAChR reconstituted into DOPC/DOPA/cholesterol in the absence of ligand (open circles), the presence of 1 mM Carb (closed squares), and the presence of 5 mM Tet (closed triangles). (B) Data were taken from spectra of the nAChR reconstituted into either DOPC/DOPA/cholesterol (closed triangles), DOPC/DOPA (closed squares), or DOPC (open circles), all in the absence of ligand.

(Figure 1) and that are very sensitive to nAChR structure and conformational change. These spectral changes were monitored for affinity-purified nAChR in the presence or absence of two ligands, Carb and Tet, and for the nAChR reconstituted into membranes either with or without cholesterol and/or anionic lipid. In membranes composed of DOPC/DOPA/cholesterol, the nAChR exists predominantly in the resting state and retains the ability to bind ligands and undergo both agonist-induced channel gating and desensitization (Fong & McNamee, 1986; McCarthy & Moore, 1992; Méthot et al., 1994). The quaternary amine local anesthetic tetracaine (Tet) appears to stabilize the resting state (Boyd & Cohen, 1984), whereas the desensitized state is stabilized by prolonged exposure to Carb (McCarthy & Moore, 1992; Méthot et al., 1994). Upon reconstitution into membranes lacking cholesterol and anionic lipids, the nAChR adopts a channel-inactive conformation that may be analogous to ligand-induced desensitization (McCarthy & Moore, 1992).

Rate and Extent of Peptide $^1H/^2H$ Exchange in the Channel-Inactive State(s). The most dramatic change in the FTIR spectra recorded after exposure of the nAChR in DOPC/DOPA/cholesterol (predominantly resting state) to ^2H_2O is a substantial decrease in the intensity of the amide II band (primarily peptide N−^{1}H bending) which is centerednear 1547 cm^{-1} in ^1H_2O, but shifts down in frequency in ^2H_2O to near 1450 cm^{-1} (Figure 1, bottom panel). The decrease in intensity is plotted as the change in the amide II/amide I ratio as a function of time of exposure to ^2H_2O in Figure 2A and provides a direct measure of the overall rates and extent of peptide $^1H/^2H$ exchange. Roughly 30% of the nAChR peptides exchange within ~5 s of exposure to ^2H_2O, 50% after 30 min, 60% after 12 h, and 75% after prolonged exposure for several days at room or lower temperatures (Baenziger & Méthot, 1995). The peptide hydrogens that exchange within seconds are highly exposed to solvent and likely involved in random and turn conformations. A large proportion of the 25% of peptides that are resistant to exchange after prolonged exposure to ^2H_2O likely correspond to α-helical peptides located within the hydrophobic, relatively solvent-inaccessible environment of the lipid bilayer (Baenziger & Méthot, 1995).

The rate of the decrease in intensity of the amide II band upon exposure of the nAChR to ^2H_2O is essentially unaffected by the presence or absence of either Tet or Carb (Figure 2A). There is still a rapid initial decrease in the intensity of the amide II band within minutes of exposure followed by a less pronounced decrease in intensity over the subsequent 12 h (Figure 2A). The extent of exchange after prolonged exposure to ^2H_2O for 12 h or 3 days (data not shown) is essentially unaffected by either ligand. The near-superimposability of the exchange data in the presence and absence of either Carb or Tet indicates that there are no large net changes in the secondary structure and/or solvent

accessibility of hydrogen-bonded structures upon desensitization. The lack of an observed change in the rates of exchange for the 25% exchange-resistant peptides, that likely reside within the hydrophobic environment of the lipid bilayer (see above), suggests that desensitization does not result from a large change in conformation of the transmembrane, ion pore-forming domains.

The overall rates of peptide $^1H/^2H$ exchange after exposure to ^2H_2O for the nAChR reconstituted into DOPC/DOPA membranes are essentially identical to those of the nAChR in DOPC/DOPA/cholesterol (Figure 2B), suggesting that there are no drastic net changes in the pattern of intramolecular peptide hydrogen bonding in the absence of cholesterol. However, upon reconstitution into membranes lacking both cholesterol and anionic lipids, an increase in the overall rates of exchange is observed (Figure 2B). The enhanced exchange is reproducibly evident within minutes of exposure to ^2H_2O as well as after prolonged exposure for either 12 h or 3 days (Figure 2B; see Figure 5 and below) and could result from a change in the secondary structure and/or an increase in the dynamics (rates and/or amplitudes of internal motions) of the nAChR. The enhanced $^1H/^2H$ exchange could also be due to an increase in the accessibility of transmembrane peptide hydrogens to ^2H_2O as a result of an increase in fluidity and thus permeability of the DOPC bilayers to aqueous solvent (see below).

The Secondary Structure-Sensitive Amide I Band in the Channel-Inactive State(s). To gain more insight into both the ligand- and lipid-dependent conformational changes, the shape of the amide I band between 1600 and 1700 cm^{-1} in spectra recorded after prolonged exposure of the nAChR to ^2H_2O was monitored in the presence and absence of the two ligands and for the nAChR reconstituted into the three different lipid membranes. The amide I band reflects primarily the peptide C=O stretching vibration and is very sensitive to hydrogen bonding and thus both protein secondary structure and peptide $^1H/^2H$ exchange. In ^1H_2O, the amide I band exhibits an intense maximum near 1655 cm^{-1} with shoulders near 1635 cm^{-1} and between 1670 and 1700 cm^{-1} (Figure 1). After 3 days in ^2H_2O at 4 °C, the resulting amide I band contour is symmetric with a relatively broad maximum between 1630 and 1660 cm^{-1} (Figures 1 and 3A). Resolution enhancement reveals a number of component bands in both ^1H_2O (not shown) and ^2H_2O (Figure 3B) that reflect the individual vibrations of peptide carbonyls in turn, β-sheet, α-helix, and random conformations. Both the assignment of the individual amide I component bands to specific secondary structures and a more detailed analysis of the secondary structural content of the nAChR have been presented elsewhere (Méthot et al., 1994; Baenziger & Méthot, 1995). The two predominant amide I component bands near 1655 and 1630 cm^{-1} in the resolution-enhanced spectra are due to peptides in α-helix and β-sheet conformations, respectively. The relatively strong intensities of both bands indicate a mixed α/β protein with a predominance of α-helical secondary structures.

The secondary structure-sensitive amide I bands in absorbance spectra of the nAChR recorded in either the presence or the absence of 1 mM Carb or 5 mM Tet are virtually superimposable as shown in Figure 3A. All spectra were recorded from the *same* nAChR sample (a film deposited on the surface of an ATR crystal), by flowing buffer containing (or lacking) Carb or Tet through the ATR

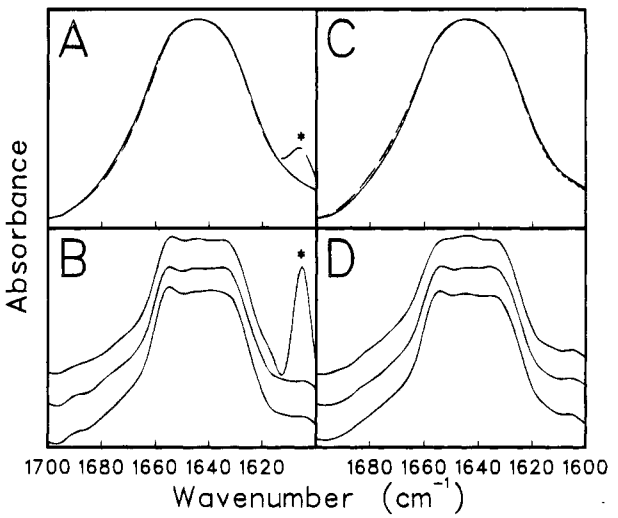

FIGURE 3: Effects of ligands and different lipids on the absorbance and deconvolved amide I band of the nAChR. Before data acquisition, the nAChR was incubated for 3 days in ^2H_2O at 4 °C. Absorbance (A) and deconvolved (B) spectra of the nAChR recorded in either the absence of ligand (solid line in panel A; bottom spectrum in panel B) or the presence of either 1 mM Carb (small dashed line in panel A; center spectrum in panel B) or 5 mM Tet (long dashed line in panel A; top spectrum in panel B). All spectra in panels A and B are from the nAChR reconstituted into DOPC/DOPA/cholesterol. The asterisk indicates an infrared absorption peak due to ligand, Tet. Absorbance (C) and deconvolved (D) spectra of the nAChR reconstituted into either DOPC/DOPA/cholesterol (solid line in panel A; bottom spectrum in panel B) or DOPC/DOPA (small dashed line in panel A; center spectrum in panel B) or DOPC (long dashed line in panel A; top spectrum in panel B).

sample compartment, which minimizes potential artifacts that can arise due to variations in sample preparation. Each exhibits a symmetric amide I contour with a broad maximum between 1630 and 1660 cm^{-1}. In all three cases, the corresponding resolution enhanced spectra exhibit the same number of component bands at essentially the same frequencies and relative intensities. Any subtle differences are typical of the variations that are observed in resolution-enhanced spectra of the nAChR recorded from different samples in the absence of ligand (compare the lower trace in both Figure 3B and Figure 3D). The similarity of the resolution-enhanced spectra indicates, in agreement with the $^1H/^2H$ exchange data, that there are no large net changes in the secondary structure of the nAChR upon desensitization. Note that the spectra were acquired to a very high signal to noise ratio and were stringently examined for the presence of residual water vapor bands (see Materials and Methods). Residual water vapor can lead to artifacts in the resolution-enhanced spectra that resemble amide I component bands and severely distort the analysis of the amide I band (Goormatigh & Ruysschaert, 1994; Reid et al., 1995).

The amide I bands in absorbance spectra of the nAChR reconstituted into lipid membranes composed of either DOPC, DOPC/DOPA, or DOPC/DOPA/cholesterol are similar as shown in Figure 3C. Resolution-enhanced spectra of the nAChR in either DOPC/DOPA or DOPC/DOPA/cholesterol are essentially identical (Figure 3D), indicating, in conjunction with the peptide $^1H/^2H$ exchange studies, that the presence or absence of cholesterol in a lipid membrane does not have a large effect on the secondary structure of the nAChR. In contrast, slight variations in the amide I band are detected upon resolution enhancement of the spectra

recorded from the nAChR reconstituted in membranes lacking both cholesterol and anionic lipids (Figure 3D). The differences between the spectra recorded from the nAChR in DOPC relative to those recorded from the nAChR in either DOPC/DOPA/cholesterol or DOPC/DOPA are reproducible with several samples prepared from different affinity purifications of the nAChR and are mainly the result of a reduced intensity of the band centered at 1655 cm^{-1} coupled with an increase in intensity between 1640 and 1650 cm^{-1}. The spectral differences could reflect a lipid-induced change in the secondary structure of the nAChR in the absence of both cholesterol and anionic lipids *that leads to* the enhanced peptide $^1H/^2H$ exchange rates noted above for the nAChR in DOPC (Figure 2B). Alternatively, given the sensitivity of the amide I band to ^2H_2O (Figure 1), the spectral differences could reflect the spectral changes that occur *as a result* of the enhanced level of peptide $^1H/^2H$. A comparison of the residual amide II band intensities indicates that an additional 5−10% of the total peptide hydrogens exchange for deuterium after 3 days of exposure of the nAChR reconstituted into DOPC to ^2H_2O relative to the nAChR in either DOPC/DOPA/cholesterol or DOPC/DOPA (see Figure 5).

Spectral Changes in the Amide I Band as a Function of Time After Exposure to ^2H_2O. In order to clarify the effects of the different lipid environments on nAChR structure and dynamics and to further explore the effects of desensitization on the structure of the nAChR, the spectral changes in the amide I band were monitored as a function of time after exposure of the nAChR to ^2H_2O. Spectral changes in the amide I band are due to the downshifts in frequency of the individual amide I component bands of peptide carbonyls in α-helix, β-sheet, turn, and random conformations upon exposure to ^2H_2O. The time course of the band shifts reflects the individual peptide $^1H/^2H$ exchange rates for each of the different types of secondary structure and provides a sensitive probe of nAChR structure and conformational change. For the nAChR in DOPC/DOPA/cholesterol, most of the changes in the shape of the amide I band are complete within seconds of exposure to ^2H_2O and are due to relatively large shifts in frequency of random coil vibrations from between 1650 and 1655 cm^{-1} in ^1H_2O down to near 1640 cm^{-1} in ^2H_2O [Figure 1 and Baenziger and Méthot (1995)]. Rapid downshifts in the frequency of relatively weak bands due to turn structures also occur in the 1670 and 1700 cm^{-1} region. Less dramatic changes in the amide I band occur between 3 min and 12 h after exposure to ^2H_2O due to the relatively small downshifts in the frequencies of α-helix and β-sheet carbonyl vibrations upon peptide $^1H/^2H$ exchange.

The frequency shifts of α-helix and β-sheet carbonyl vibrations are detected in resolution-enhanced spectra recorded as a function of time after exposure of the nAChR to ^2H_2O (not shown). The individual band shifts of α-helix and β-sheet are reflected by the downshift of intensity above 1660 cm^{-1} and below 1640 cm^{-1}, and by the decrease in the intensity near 1655 cm^{-1} coupled with an increase in intensity between 1640 and 1650 cm^{-1}, respectively. In addition, the band shifts can be seen more clearly in exchange difference spectra calculated by subtracting the spectra recorded at various time points from the spectrum recorded after 3 min of exposure to ^2H_2O (Figure 4). The individual band shifts of β-sheet secondary structures are reflected by the negative and positive bands near 1680 and 1630 cm^{-1}

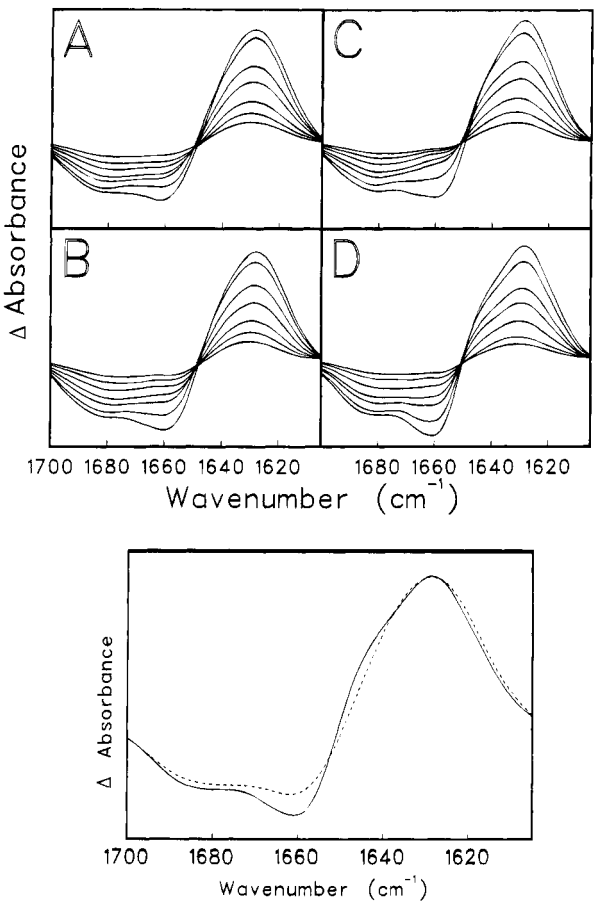

FIGURE 4: Exchange difference spectra showing the spectral shifts that occur in the amide I band upon exposure of the nAChR to ^2H_2O. In each case, the spectrum recorded after 3 min of exposure to ^2H_2O was subtracted from spectra recorded after 6, 9, 16, 40, 80, 600, and 780 min of exposure to ^2H_2O. All spectra were base line corrected between 1713 and 1604 cm^{-1}. The presented spectra are the average of at least two different exchange experiments. (A) nAChR in DOPC/DOPA/cholesterol in the absence of ligand; (B) nAChR in DOPC/DOPA/cholesterol in the presence of 1 mM Carb; (C) nAChR in DOPC/DOPA in the absence of ligand; and (D) nAChR in DOPC in the absence of ligand. Bottom panel: superposition of the exchange difference spectrum calculated at 12 h for the nAChR in DOPC (solid line) over the same difference spectrum recorded from the nAChR in DOPC/DOPA/cholesterol (dashed line). All spectra were smoothed using a Savitsky−Golay algorithm (GRAMS, Galactic Industries; Salem, NH) with a polynomial of 3 and 15 smoothed points.

(Figure 4). Other β-sheet bands may be masked by overlapping bands due to the exchange of α-helical secondary structures. Those of α-helix are reflected by a negative band near 1660 cm^{-1} coupled with the positive shoulder near 1645 cm^{-1}. The latter is partially obscured by the more intense band near 1630 cm^{-1}, but can be seen clearly in exchange difference spectra recorded from the nAChR in DOPC (Figure 4D).

The exchange difference spectra calculated from spectra recorded in the presence and absence of Carb are essentially superimposable as shown in Figure 4A,B. In both cases, the majority of peptides in β-sheet conformations exchange within the first 2 h of exposure to ^2H_2O whereas the α-helical structures exchange on a much slower time scale. The exchange difference spectra indicate that both the rates and extent of exchange of α-helix and β-sheet peptide hydrogens are individually unaffected by Carb and, in conjunction with both the secondary structure analysis and the bulk peptide

$^1H/^2H$ exchange rates monitored above, provide compelling evidence that Carb does not lead to a large change in the structure or dynamics of the nAChR. In addition, the lack of a change in the intensities of bands near 1660 and 1645 cm^{-1} in the exchange difference spectra recorded after 12 h of exposure (Figure 4), and of the band near 1655 cm^{-1} in the resolution-enhanced spectra after prolonged exposure to ^2H_2O for 3 days (Figure 3), suggests further that there is no change in the rates of exchange of the otherwise exchange-resistant α-helical secondary structures likely found within the hydrophobic environment of the lipid bilayer. Desensitization must result from a very subtle change in structure upon exposure of the nAChR to cholinergic agonists and appears to have no dramatic effect on the transmembrane domains of the nAChR.

The rates of the spectral changes in the amide I band upon exposure to ^2H_2O are similar for the nAChR reconstituted in membranes composed of either DOPC/DOPA/cholesterol or DOPC/DOPA (Figure 4), suggesting, in agreement with the above data, that cholesterol is not required to stabilize the native secondary structure of the nAChR. In contrast, the exchange difference spectra recorded from the nAChR in DOPC reveal an increased intensity of the coupled negative and positive bands near 1660 and 1645 cm^{-1}, indicating an enhanced exchange of otherwise exchange-resistant α-helical peptides. The increased intensity of both bands is reproducible in several experiments and is particularly evident when the difference spectra calculated at 12 h for the nAChR in DOPC are superimposed on the same difference spectra calculated for the nAChR in DOPC/DOPA/cholesterol (Figure 4, bottom panel). Similarly, resolution-enhanced spectra recorded after 3 days exposure to ^2H_2O reveal a more dramatic decrease in intensity of the amide I component band near 1655 cm^{-1} corresponding to an increased exchange of α-helical structures (Figures 3D and 4). These results suggest that the above noted changes in the resolution-enhanced amide I band upon reconstitution into DOPC *are the result* of the enhanced peptide $^1H/^2H$ exchange of α-helical structures and may not reflect a change in secondary structure of the nAChR.

To test whether the enhanced peptide $^1H/^2H$ exchange is responsible for the spectral changes in the amide I band of the nAChR in DOPC relative to the nAChR in DOPC/DOPA/cholesterol, we compared the two resolution-enhanced amide I bands with the resolution-enhanced amide I bands observed in spectra of the nAChR in DOPC recorded after 2 and 12 h of exposure to ^2H_2O. An additional 10% of the peptide hydrogens exchange for deuterium for the nAChR in DOPC relative to the nAChR in DOPC/DOPA/cholesterol, both after 3 days in ^2H_2O (Figure 5A). The difference in the extent of exchange between the nAChR in DOPC after 2 and 12 h of exposure to ^2H_2O is also ~10% (Figure 5B), although the total number of exchanged peptide hydrogens is less than the number exchanged after 3 days in ^2H_2O (in either DOPC or DOPC/DOPA/cholesterol). The spectral differences between the resolution-enhanced amide I band for the nAChR in DOPA/DOPA/cholesterol relative to the nAChR in DOPC (Figure 5C) are very similar to the differences observed between spectra of the nAChR in DOPC recorded during the 2nd relative to the 12th h of peptide $^1H/^2H$ exchange (Figure 5D). As the latter are due entirely to an enhanced level of peptide $^1H/^2H$ exchange, it can be concluded that the increase in the rates and thus extent of

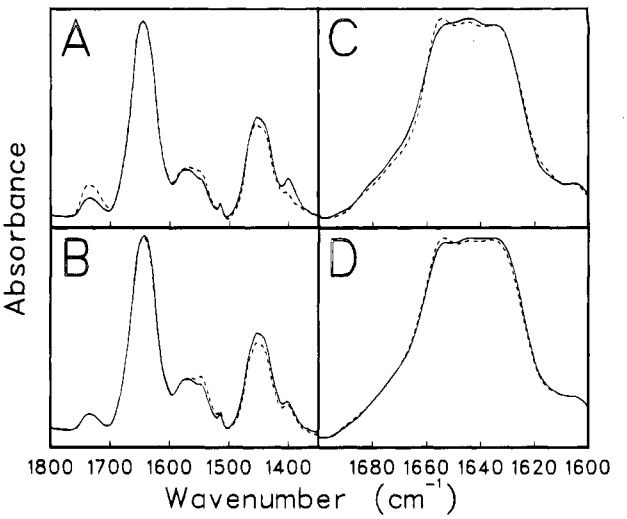

FIGURE 5: Comparison of the differences between absorbance and resolution-enhanced spectra of the nAChR reconstituted into DOPC/DOPA/cholesterol and in DOPC with the differences in spectra of the nAChR in DOPC recorded 2 and 12 h after exposure to ^2H_2O. (A) Absorbance and (C) resolution-enhanced spectra of the nAChR in DOPC/DOPA/cholesterol (dashed line) and in DOPC (solid line) both after 3 days exposure to ^2H_2O at 4 °C. (B) Absorbance and (D) resolution-enhanced spectra of the nAChR in DOPC after 2 (dotted line) and 12 (solid line) h of exposure to ^2H_2O. The spectra are base line corrected between 1800 and 1350 cm^{-1}. The two spectra in both panel A and panel C are normalized according to the relative intensities of the amide I band. The spectra in panels B and D are not normalized.

peptide $^1H/^2H$ exchange in DOPC is responsible for the spectral changes observed in the resolution-enhanced amide I band of the nAChR in DOPC. The lack of spectral changes, in addition to those expected due to the enhanced peptide $^1H/^2H$ exchange, suggests that there are no large net changes in the secondary structure of the nAChR upon reconstitution into membranes lacking both cholesterol and anionic lipids. The increase in exchange rates must therefore be due to an increase in the dynamics of the nAChR and/or an increased accessibility of the transmembrane α-helices to ^2H_2O as a result of an increase in the "fluidity" and thus the permeability of the DOPC bilayers (see Discussion).

DISCUSSION

The main goal of the present study was to clarify the nature of the structural changes that lead to the formation of the channel-inactive state(s) of the nAChR upon either prolonged exposure to agonist (desensitization) or reconstitution into synthetic lipid membranes lacking cholesterol and anionic lipids. Several studies using a wide variety of techniques have been unable to detect a global change in structure upon Carb-induced desensitization (Mielke & Wallace, 1988; McCarthy & Stroud, 1989; Wu et al., 1990; Butler & McNamee, 1993; Aslanian et al., 1993; Méthot et al., 1994). The Carb-induced conformational change in the peptide backbone detected by FTIR difference spectroscopy is restricted to relatively few amino acid residues (Baenziger et al., 1993). In contrast, a substantial rearrangement in the secondary structure of affinity-purified nAChR has been detected by others both in the presence of Carb (Castresana et al., 1992; Fernandez-Ballester et al., 1994) and upon reconstituted into lipid membranes lacking either cholesterol and/or anionic lipids (Fong & McNamee, 1987; Butler & McNamee, 1993; Fernandez-Ballester et al., 1994). As the

nAChR appears to be stabilized in a desensitized state in the absence of these two lipids (McCarthy & Moore, 1992), the latter studies imply more substantial changes in the secondary structure of the desensitized nAChR.

To clarify the structural changes responsible for channel inactivation and thus provide a firm basis for understanding the mechanism(s) of desensitization, we examined the structure of the nAChR in the various channel-inactive states by recording FTIR spectra both as a function of time and after prolonged exposure of the nAChR to ^2H_2O. FTIR spectra recorded as a function of time after exposure to ^2H_2O reveal a number of spectral changes in both the amide I and amide II bands that provide a direct measure of the overall rates and extent of exchange of peptide hydrogens for deuterium, as well as insight into the individual rates and extent of exchange of specific secondary structures, such as α-helix and β-sheet (Heimburg & Marsh, 1993; Baenziger & Méthot, 1995). The shape of the resolution-enhanced amide I band in spectra recorded after prolonged exposure to ^2H_2O is also sensitive to the secondary structure of the nAChR. The overall rates of exchange, the individual rates of exchange for α-helix and β-sheet peptide hydrogens, and the percentage of unexchanged peptides after prolonged exposure to ^2H_2O for up to several days at 4 °C are all essentially identical in the presence and absence of both Carb and Tet. The resolution-enhanced amide I bands in spectra recorded in the presence of either Carb or Tet are also virtually superimposable onto the amide I band recorded in the absence of either ligand. The results provide compelling evidence that desensitization is not accompanied by a global change in structure of the nAChR.

The hydrogen exchange kinetics and amide I band contour monitored for the nAChR reconstituted into either DOPC/DOPA or DOPC/DOPA/cholesterol are essentially identical, indicating that the absence of cholesterol in the reconstituted nAChR membrane has no detectable effect on the global structure of the nAChR. In contrast, although the secondary structure of the nAChR appears to be unaffected by reconstitution into membranes lacking both cholesterol and anionic lipids (i.e., DOPC), there is a slight increase in the rates of peptide $^1H/^2H$ exchange. The enhanced exchange is due to an overall increase in the rates and thus extent of exchange of the otherwise exchange-resistant α-helical peptide hydrogens that are likely found within the hydrophobic environment of the lipid bilayer (Baenziger & Méthot, 1995). The enhanced peptide $^1H/^2H$ exchange could be due to a change in the amplitudes and/or rates of the internal motions of the nAChR. The increased level of exchange could also be due to an increased permeability of the lipid bilayer and thus an increased accessibility of the transmembrane domains to ^2H_2O.

Cholesterol has a well-characterized condensing effect on lipid bilayers in the liquid crystalline phase and leads to a relatively tightly packed or ordered hydrophobic interior (Smith, 1984) that is less permeable to aqueous solvent (Carruthers & Melchior, 1983). Similarly, the very small head group of DOPA could lead to a condensation of the lipid bilayer surface area, an increase in order of the lipid acyl chains, and a corresponding decrease in the solvent accessibility of the transmembrane α-helices to aqueous solvent [for a discussion of the effects of lipid head group size on acyl chain order, see page 2606 of Salmon et al. (1987) and also see Marcelja (1974)]. The absence of this condensing or ordering effect on the hydrophobic environment in the bilayers composed of only DOPC may account for the increased accessibility of the putative transmembrane α-helices to ^2H_2O and thus an increase in peptide $^1H/^2H$ exchange with a concomitant change in the amide I band.

The hydrogen/deuterium exchange spectra suggest further that channel inactivation does not result from a large perturbation of the structure of the transmembrane pore-forming domains of the nAChR. A large proportion of the 25% of the total nAChR peptides that remain unexchanged after prolonged exposure of the nAChR to ^2H_2O is likely found within the hydrophobic environment of the lipid bilayer [see Baenziger and Méthot (1995)]. The similarity in the extent of exchange after prolonged exposure of the nAChR to ^2H_2O (as judged by monitoring the residual amide II band intensity and the changes in shape of the amide I contour) in the presence and absence of Carb and Tet indicates that desensitization does not significantly alter the rates of exchange of these putative exchange-resistant transmembrane α-helical peptides. The similarity in the exchange data in the presence and absence of cholesterol also suggests that cholesterol does not alter the secondary structure of the transmembrane domains, as has been suggested by others (Fong & McNamee, 1987). Chemical labeling studies using the hydrophobic, photoactivatable probe, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine (TID), indicate that the exposure of most of the hydrophobic amino acid side chains in the putative transmembrane α-helices to the hydrophobic environment of the lipid bilayer is essentially unchanged in the presence of Carb (Blanton & Cohen, 1994). In addition, the majority of the amide I bands that are detected in FTIR resting-to-desensitized difference spectra and that reflect the subtle conformational change in the polypeptide backbone associated with desensitization undergo a rapid downshift in frequency upon exposure of the nAChR to ^2H_2O (Baenziger et al., 1995; Baenziger and Chew, unpublished results). The subtle peptide backbone conformational change responsible for desensitization may therefore involve predominantly amino acid residues that are highly accessible to the aqueous solvent.

Alternatively, our data are compatible with a slight twisting and/or a change in orientation of the transmembrane domains that does not significantly affect either the secondary structure or the peptide hydrogen−deuterium exchange rates of the transmembrane domains. Unwin recently attributed a rotational change in the pore-lining transmembrane α-helices detected in the 9 Å electron density map to the gating of the nAChR ion channel (Unwin, 1995). More substantial rotations in the transmembrane segments were detected in lower resolution data upon prolonged exposure to Carb and thus desensitization (Unwin, 1995). In addition, the labeling pattern of the nAChR by a hydrophobic photoactivatable cholesterol analog is sensitive to Carb, suggesting a change in the exposure of transmembrane amino acid side chains to the hydrophobic probe (Fernandez et al., 1993). The latter could be due to an altered binding of the cholesterol analog to "non-annular" cholesterol binding sites.

The origin of the discrepancy between our FTIR data, which suggest that both ligand- and lipid-dependent channel inactivation are due to very subtle changes in nAChR structure, and the various FTIR studies, which have reported dramatic ligand and lipid changes in secondary structure, is currently unclear, but could reflect different methods of

sample preparation. The amide I band analysis presented here was performed on spectra acquired from nAChR samples previously equilibrated with ^2H_2O for 3 days at 4 °C. After 3 days in ^2H_2O, roughly 75% of the nAChR peptide hydrogens exchange for deuterium, and prolonged exposure to ^2H_2O has little effect on either the extent of exchange or the consequent shape of the amide I band (Baenziger & Méthot, 1995). In contrast, both Castresana et al. (1992) and Fernandez-Ballester et al. (1994) analyzed the amide I band in FTIR spectra recorded from the nAChR in the presence and absence of Carb and reconstituted into lipid membranes lacking cholesterol after only relatively brief centrifugation resuspension cycles in ^2H_2O buffer.

Roughly 30% of the peptide hydrogens exchange within 5 s of exposure of the nAChR to ^2H_2O and an additional 30% between 5 s and 12 h (Figures 1 and 2; Baenziger & Méthot, 1995). The increasing levels of peptide $^1H/^2H$ exchange over the first 12 h of exposure to ^2H_2O lead to variations in the shape of the amide I band (Figure 1, see bottom panel) that are similar to the spectral changes reported by Castresana et al. (1992) and Fernandez-Ballester et al. (1994) upon the addition of Carb to the nAChR and upon reconstitution into lipid membranes lacking cholesterol. As the spectra of both Castresana et al. (1992) and Fernandez-Ballester et al. (1994) were likely recorded over this time period when spectral changes in the amide I band due to increasing levels of peptide $^1H/^2H$ exchange should occur, some of their conclusions may have been influenced by sample variations in the levels of peptide $^1H/^2H$ exchange. Similarly, the more subtle changes in shape of the amide I band that have been reported by Butler and McNamee (1993) upon reconstitution into different lipid membranes may reflect the effects of minor variations in the exchange rates in the absence of cholesterol and/or anionic lipids. In light of the sensitivity in the shape of the amide I band to varying levels of peptide $^1H/^2H$ exchange, the substantial lipid- and ligand-dependent changes in secondary structure of the nAChR reported by others may need to be revised. Both lipid- and ligand-dependent modulation of nAChR function are likely the result of subtle changes in structure, as is the case in the modulation of the function of other allosteric proteins, such as hemoglobin (Perutz, 1990).

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