

Lipid-Protein Interactions at the Nicotinic Acetylcholine Receptor

A FUNCTIONAL COUPLING BETWEEN NICOTINIC RECEPTORS AND PHOSPHATIDIC ACID-CONTAINING LIPID BILAYERS*

Received for publication, August 29, 2001, and in revised form, October 24, 2001
Published, JBC Papers in Press, October 26, 2001, DOI 10.1074/jbc.M108341200

Corrie J. B. daCosta[‡], Andrei A. Ogresl[‡], Elizabeth A. McCardy[§], Michael P. Blanton[§],
and John E. Baenziger[‡]¶

From the [‡]Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada and the [§]Departments of Anesthesiology and Pharmacology, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

The structural and functional properties of reconstituted nicotinic acetylcholine receptor membranes composed of phosphatidyl choline either with or without cholesterol and/or phosphatidic acid have been examined to test the hypothesis that receptor conformational equilibria are modulated by the physical properties of the surrounding lipid environment. Spectroscopic and chemical labeling data indicate that the receptor in phosphatidylcholine alone is stabilized in a desensitized-like state, whereas the presence of either cholesterol or phosphatidic acid favors a resting-like conformation. Membranes that effectively stabilize a resting-like state exhibit a relatively large proportion of non-hydrogen-bonded lipid ester carbonyls, suggesting a relatively tight packing of the lipid head groups and thus a well ordered membrane. Functional reconstituted membranes also exhibit gel-to-liquid crystal phase transition temperatures that are higher than those of nonfunctional reconstituted membranes composed of phosphatidylcholine alone. Significantly, incorporation of the receptor into phosphatidic acid-containing membranes leads to a dramatic increase in both the lateral packing densities and the gel-to-liquid crystal phase transition temperatures of the reconstituted lipid bilayers. These results suggest a functional link between the nicotinic acetylcholine receptor and the physical properties of phosphatidic acid-containing membranes that could underlie the mechanism by which this lipid preferentially enhances receptor function.

Many hypotheses have been suggested to account for the functional sensitivity of the nicotinic acetylcholine receptor (nAChR)¹ from *Torpedo* to the lipid composition of its surrounding environment (1, 2). Roles for bulk membrane fluidity (3), specific lipid binding sites (4, 5), and annular lipids (6, 7)

have all been proposed. Despite numerous studies aimed at understanding lipid-protein interactions at the nAChR, however, there is still disagreement even as to which lipids are absolutely required for optimal function. Consequently, the molecular mechanisms by which lipids modulate function remain poorly understood. The lack of definitive insight reflects both the complexities of lipid-protein interactions at the nAChR and the inherent difficulties associated with characterizing the structural and dynamic properties of the nAChR and its surrounding lipids in a membrane environment.

The pioneering work of the Barrantes and McNamee laboratories originally highlighted the requirement for anionic and/or neutral lipids such as phosphatidic acid and cholesterol (Chol), respectively, in reconstituted phosphatidylcholine membranes to stabilize a functional nAChR (3, 8, 17). It was suggested that phosphatidic acid and Chol stabilize distinct β -sheet and α -helix structures, respectively (4, 9–11). In contrast, more recent studies indicate that lipids exert their effects on nAChR function through subtle structural alterations (12, 13). The nAChR in egg phosphatidylcholine (EPC) membranes lacking both neutral and anionic lipids appears to be stabilized in a nonconducting desensitized-like conformation (5, 14). Increasing levels of either Chol or dioleoyl phosphatidic acid (DOPA) in EPC membranes stabilize an increasing proportion of nAChRs in a resting-like state that is capable of undergoing agonist-induced conformational change (5). Both lipids also slow nAChR internal motions and increase the lateral packing density of the lipid bilayers (15). These findings were incorporated into the model of lipid action at the nAChR presented in Fig. 1 (5). A basic tenet of the model is that the equilibrium between the resting and the nonconducting desensitized states is modulated by bulk physical properties of the lipid bilayer. Specific anionic lipid binding sites are also likely important (see Ref. 5 for a detailed discussion of the model).

The hypothesis that membrane physical properties influence nAChR function is not new, but there is contradictory evidence both in support of and against such a mechanism of lipid action (3, 11, 15, 16). As we have suggested previously (5), this controversy may stem from the fact that studies testing the link between fluidity and function have generally relied on spin label and fluorescent probes to assess the physical properties of the reconstituted nAChR membranes. Both approaches have characterized the reconstituted membranes in terms of a single parameter, which may not be sufficient considering the complex motional and dynamic properties of lipid bilayers. A combination of solid state ²H NMR spectroscopy and molecular modeling can provide a more comprehensive picture of the motions and dynamics of lipids in a membrane environment and could lead to a more accurate assessment of the link

* This work was supported in part by a grant from the Canadian Institutes of Health Research (to J. E. B.) and National Institutes of Health NINDS Grant NS35786 (to M. P. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, Microbiology, and Immunology, University of Ottawa, 451 Smyth Rd., Ottawa, Ontario K1H 8M5, Canada. Tel.: 613-562-5800 (ext. 8222); Fax: 613-562-5440; E-mail: jebaenz@uottawa.ca.

¹ The abbreviations used are: nAChR, nicotinic acetylcholine receptor; EPC, egg phosphatidylcholine; DOPA, dioleoyl phosphatidic acid; DOPC, dioleoyl phosphatidylcholine; POPA, 1-palmitoyl-2-oleoyl phosphatidic acid; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; Chol, cholesterol; Carb, carbamylcholine; TID, 3-trifluoromethyl-3-(*m*-iodophenyl)diazirine; FTIR, Fourier transform infrared; MOPS, 4-morpholinopropanesulfonic acid.

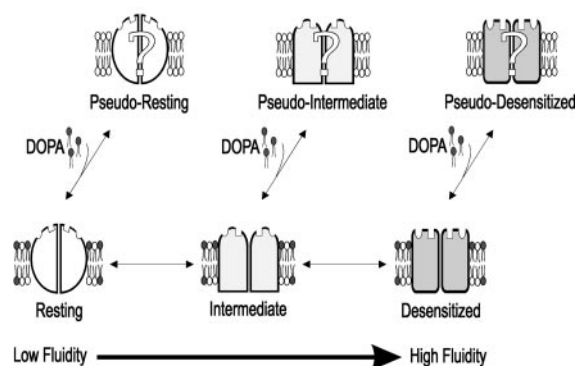


FIG. 1. A speculative model of lipid-protein interactions at the nAChR. The model suggests that membrane fluidity modulates nAChR conformational equilibria with a relatively fluid membrane stabilizing a desensitized-like conformation and a membrane of low fluidity stabilizing a resting-like state. Anionic lipids such as DOPA and POPA are required for the nAChR to adopt a fully functional conformation. Conformational states intermediate between the resting and desensitized states are also possible (5).

between fluidity and nAChR function (18, 19). ^2H NMR spectroscopy requires membrane lipids with either perdeuterated or single-site deuterium-labeled fatty acyl chains. Lipids such as 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl phosphatidic acid (POPA) perdeuterated along the saturated palmitoyl chain are commercially available and are ideal for such a study because interpretation of the ^2H quadrupolar splittings is not complicated by the orientational constraints of a double bond (32). Unfortunately, ^2H NMR/molecular modeling studies are extremely time-consuming and require the acquisition and analysis of extensive NMR data. The structural and functional properties of the nAChR in mixtures of POPC, POPA, and Chol have also not been defined.

As a first step toward rigorously testing the hypothesized link between nAChR conformational equilibria and the physical properties of the lipid environment surrounding the nAChR, we characterize here the structural and functional properties of reconstituted nAChR membranes composed of POPC, 3:2 POPC/POPA, 3:2 POPC/1,2-dioleoyl phosphatidic acid (DOPA), 3:2 POPC/Chol, and 3:1:1 POPC/POPA/Chol using both Fourier transform infrared (FTIR) spectroscopy and chemical labeling techniques. These lipid mixtures were chosen because each lipid is available in a deuterated form and because preliminary studies suggested that the mixtures should stabilize the nAChR predominantly in either a resting-like (3:2 POPC/POPA, 3:2 POPC/DOPA, 3:2 POPC/Chol, and 3:1:1 POPC/POPA/Chol) or a desensitized-like (POPC) state. As Chol, DOPA, and POPA have distinct structures and likely distinct effects on membrane physical properties, these mixtures provide simple systems in which potential links between the membrane environment and nAChR function can be rigorously tested.

We have characterized the physical properties of the reconstituted nAChR membranes using FTIR spectroscopy, which provides detailed, but qualitative insight into the motional properties of membrane lipids (35). The FTIR data are consistent with a modulation of nAChR conformational equilibria by membrane fluidity, but show that the physical properties of even these simple reconstituted membranes are complex. Significantly, the data indicate that the nAChR selectively influences the physical properties of the lipid environment in which it is imbedded when either DOPA or POPA is present. This novel finding provides direct evidence for a coupling between the physical properties of phosphatidic acid-containing membranes and the functional state of the nAChR that could un-

derlie a mechanism by which lipids modulate nAChR function. The structural and functional characterization of the nAChR in these lipid mixtures also provides a basis for future ^2H NMR/molecular modeling studies aimed at further testing the link between fluidity and function.

EXPERIMENTAL PROCEDURES

Materials—Frozen *Torpedo californica* electroplax tissue was obtained from either Marinus (Long Beach, CA) or Aquatic Research Consultants (San Pedro, CA). POPC, POPA, and DOPA were from Avanti Polar Lipids, Inc. (Alabaster, AL) and both cholesterol and carbamylcholine (Carb) were from Sigma. 3-Trifluoromethyl-3-(m - ^{125}I iodophenyl) diazine (^{125}I TID; ~ 10 Ci/mmol) was obtained from Amersham Biosciences, Inc. and stored in ethanol at 4 °C.

nAChR Purification and Reconstitution—The nAChR was affinity-purified on a bromoacetylcholine bromide-derivatized Affi-Gel 102 column (Bio-Rad) as described previously (14), but with several modifications. For each reconstitution, crude nAChR membranes from roughly 100 g of *Torpedo* electroplax tissue were solubilized for 1 h at 4 °C in a total volume of 100 ml of dialysis buffer (100 mM NaCl, 10 mM Na_2PO_4 , 0.1 mM EDTA, 0.02% w/v NaN_3 , pH = 7.8) containing 1% cholate. The solubilized membranes were centrifuged for 30 min at $87,000 \times g$ to pellet insoluble material and the supernatants applied to a 10-ml affinity column at a flow rate of 1 ml/min. The column was then washed with 32.5 ml of a 1.3 mM lipid solution in 1% cholate dialysis buffer. This was followed by a 15-ml linear gradient to a 3.2 mM lipid solution in 1% cholate dialysis buffer and an additional 15-ml wash to facilitate complete exchange of endogenous for defined lipids. A 15-ml linear gradient to a 0.13 mM lipid solution in 0.5% cholate dialysis buffer was followed by a 35-ml wash. The nAChR was then eluted with a 0.13 mM lipid solution in 250 mM NaCl, 0.1 mM EDTA, 0.02% NaN_3 , 5 mM phosphate, pH 7.8, with 0.5% cholate and 10 mM Carb. All lipid washes were at 2 ml/min under the control of a fast protein liquid chromatograph (Amersham Biosciences AB, Uppsala, Sweden). Fractions with an A_{280} greater than 0.05 were pooled in dialysis bags (12,000–14,000-Da cut-off) and dialyzed five times against 2 liters of dialysis buffer with buffer change once every 12 h.

The dialyzed membranes were centrifuged at $120,000 \times g$ for 2 h to pellet the reconstituted membranes. Yields were typically 6–10 mg of nAChR protein as determined by BCA assay (Pierce). The purity of all nAChR samples were analyzed by 12% SDS-PAGE with Coomassie Blue staining. Lipid-protein molar ratios were calculated by FTIR (20) and were generally found to be in the 140–180:1 molar range (Table II). The final lipid composition of each reconstituted membrane was assessed by thin layer chromatography using Silica gel 60 WF₂₅₄ aluminum sheets from Merck (Darmstadt, Germany) in $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$, 65/25/5, v/v/v. Lipids were visualized with a 0.05% w/v solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 90:5 v/v water/glacial acetic acid, followed by heating at 100 °C. The final lipid composition of each reconstituted sample did not vary qualitatively from control lipid mixtures. No endogenous lipids were detected.

Transmission FTIR Spectroscopy—All FTIR spectra were recorded on either a Bio-Rad FTS 575 or a FTS 40 spectrometer (Randolph, MA), both equipped with a DTGS detector. For each sample, 250 μg of reconstituted nAChR in 50 μl of 2 mM $^1\text{H}_2\text{O}$ phosphate buffer (pH 7.0) was centrifuged and the pellet resuspended in 300–400 μl of 2 mM $^2\text{H}_2\text{O}$ phosphate buffer, pH 7.0. After repeating once, the final $^2\text{H}_2\text{O}$ suspensions were incubated at 4 °C for precisely 72 h to exchange peptide N^1H for N^2H and then stored at -80 °C. Prior to FTIR analysis, samples were individually thawed, centrifuged, and resuspended in 30 μl of $^2\text{H}_2\text{O}$ phosphate buffer. After five freeze-thaw vortex cycles, each was deposited on a 1-cm diameter CaF_2 window and the excess buffer evaporated with a gentle stream of dry nitrogen. The nAChR film was rehydrated with 8 μl of Torpedo Ringer buffer in $^2\text{H}_2\text{O}$ (pD 7.0) and sandwiched between a second CaF_2 window with a 12- μm Teflon spacer. The sandwich was placed in a thermostatically controlled transmission cell from Harrick Scientific (Ossining, NY) and spectra recorded at 2 cm^{-1} resolution signal averaging 4000 scans. Spectra were analyzed using GRAMS/32 v.5.01 software (Galactic, Salem, NH) to test for and if necessary subtract uncompensated water vapor (31). Deconvolution of the amide I and lipid carbonyl stretching bands was performed with $\gamma = 10.0$ and a smoothing parameter of 70%.

Thermotropic Phase Behavior—Reconstituted nAChR membranes prepared as described above were placed in the thermostatic transmission cell equilibrated with circulating water from an RTE-110 water bath/circulator (Neslab, Newington, NH). Spectra (2 cm^{-1} resolution and 128 scans) were recorded at 1 °C intervals as the sample was cooled

from 35 to -10°C using the software program DeltaTemp from Neslab. The actual temperature of the sample cell was monitored using an electronic thermometer (Barnant, Barrington, IL) with a type J thermocouple probe. A 5-min time interval was allowed for the water bath to equilibrate at each temperature and then another 10 min for sample equilibration.

FTIR Difference Spectroscopy—FTIR difference spectra were recorded at 22.5°C using the attenuated total reflectance technique as described in detail elsewhere (21, 22). All spectra were recorded at 8 cm^{-1} resolution signal averaging 512 scans/spectrum. Each presented spectrum is the average of between 35 and 70 individual difference spectra recorded from at least two different films from at least two separate reconstitutions. The difference spectra were base line-corrected between 1800 and 1000 cm^{-1} and were interpolated to an effective resolution of 4 cm^{-1} .

Photolabeling with $[^{125}\text{I}]\text{TID}$ —nAChR conformation and agonist-induced state transitions were assessed by the technique of hydrophobic photolabeling with $[^{125}\text{I}]\text{TID}$ as described in detail elsewhere (14, 26–28). Briefly, an aliquot containing $250\text{ }\mu\text{g}$ of nAChR protein from each affinity-purified membrane was incubated at a protein concentration of 0.227 mg/ml in a $0.4\text{ }\mu\text{M}$ $[^{125}\text{I}]\text{TID}$ solution (10 mM MOPS, 100 mM NaCl, 0.1 mM EDTA, and 0.02% NaN_3 , pH 7.5) either with or without $400\text{ }\mu\text{M}$ Carb. After 2 h at room temperature under reduced lighting conditions, each sample was irradiated with a 365-nm UV lamp (Spectroline EN-280L) for 7 min at a distance of less than 1 cm. The membranes were centrifuged at $39,000 \times g$ for 1 h and the resulting pellets solubilized in electrophoresis sample buffer. Individual nAChR subunits were separated by SDS-PAGE using 1.0-mm -thick gels. The separating gel was composed of 8% polyacrylamide, 0.33% bis-acrylamide. The gels were stained with Coomassie Blue R-250 to visualize nAChR subunit bands and were then dried and exposed to Kodak X-Omat LS film with an intensifying screen at -80°C ($2\text{--}18\text{ h}$ of exposure). $[^{125}\text{I}]\text{TID}$ incorporation into each nAChR subunit was quantified by cutting out the receptor bands from the dried 8% acrylamide gel and then assessing the amount of ^{125}I cpm in each band by γ -counting in a Packard Cobra II γ counter (10 min of counting time/band).

RESULTS

nAChR Structure and Internal Dynamics—FTIR spectra of the nAChR recorded in $^2\text{H}_2\text{O}$ buffer exhibit two intense protein bands that are sensitive to protein structure and dynamics (23). The amide I band between 1600 and 1700 cm^{-1} reflects predominantly the carbonyl stretching vibration of the polypeptide backbone, and its shape is highly sensitive to protein secondary structure. The amide I band shapes observed in spectra of the nAChR reconstituted into membranes composed of POPC, 3:2 POPC/Chol, 3:2 POPC/POPA, 3:2 POPC/DOPA, and 3:1:1 POPC/POPA/Chol are all similar. Deconvolution shows that each exhibits the same number of component bands with similar frequencies and relative intensities. The similarities of the deconvolved spectra indicate, in contrast to earlier studies (3, 9), that the secondary structure of the nAChR is essentially unaffected by the presence or absence of neutral and anionic lipids.

Close inspection of the resolution enhanced spectra, however, reveals that the presence of Chol and/or either POPA or DOPA in the POPC membranes leads to a subtle increase in intensity of the α -helix component band near 1655 cm^{-1} and a slight decrease in intensity between 1640 and 1650 cm^{-1} . α -Helical vibrations shift down from 1655 cm^{-1} to between 1640 and 1650 cm^{-1} upon peptide $^1\text{H}/^2\text{H}$ exchange (24, 25). The slight increase in intensity near 1655 cm^{-1} in the presence of Chol and/or POPA or DOPA could reflect a slight increase in the proportion of α -helical peptides that remain in a protiated versus a deuterated form after 3 days of exposure to $^2\text{H}_2\text{O}$. In agreement with this interpretation, the residual amide II intensity between 1520 and 1580 cm^{-1} , which is directly related to the number of unexchanged peptide hydrogens, is slightly more intense in spectra of the nAChR reconstituted into POPC membranes containing Chol and/or POPA or DOPA than in spectra of the nAChR reconstituted into POPC alone (Fig. 2, right panel). The increased amide II band intensity suggests a

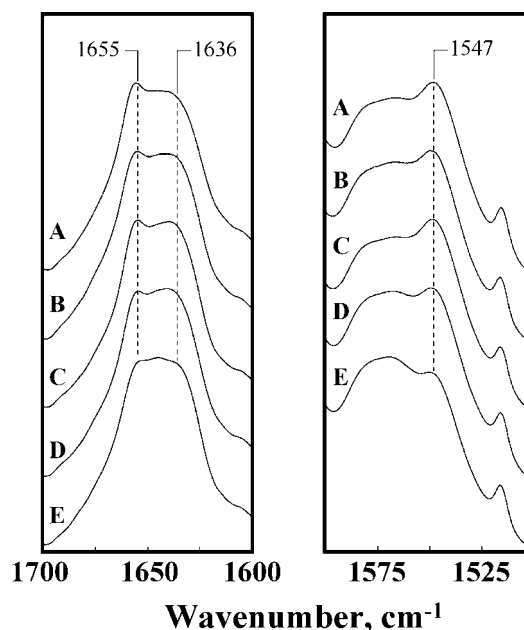


FIG. 2. The deconvolved amide I band (left panel) and residual amide II band intensity (right panel) in FTIR spectra recorded from the nAChR reconstituted into 3:1:1 POPC/POPA/Chol (A), 3:2 POPC/DOPA (B), 3:2 POPC/POPA (C), 3:2 POPC/Chol (D), and POPC (E). The amide II bands presented in the right panel are not deconvolved. All samples were exposed to $^2\text{H}_2\text{O}$ buffer for 72 h at 4°C prior to data acquisition. A spectrum of $^2\text{H}_2\text{O}$ buffer has been subtracted from each.

slowing of peptide $^1\text{H}/^2\text{H}$ exchange in POPC membranes that contain Chol and/or POPA or DOPA (see “Discussion”).

nAChR Conformational Equilibria—The effect of lipid composition on nAChR conformational equilibria was first examined using FTIR difference spectroscopy. The difference between spectra of the nAChR recorded in the presence and absence of Carb (referred to as a Carb difference spectrum) exhibits five positive marker bands centered near 1663 , 1655 , 1547 , 1430 , and 1059 cm^{-1} (the latter two not shown). These marker bands, which reflect vibrational changes associated with the resting to desensitized conformational transition (13, 22), are evident in difference spectra recorded from the nAChR reconstituted into 3:1:1 POPC/POPA/Chol, 3:2 POPC/POPA, and 3:2 POPC/DOPA membranes (Fig. 3). The positive intensity indicates that each membrane stabilizes a resting-like state that is capable of undergoing agonist-induced conformational change. In contrast, positive intensity at each of the five marker frequencies is absent in difference spectra recorded from reconstituted POPC membranes suggesting that the nAChR cannot respond to agonist binding and is likely stabilized in a desensitized state (see below). The nAChR in 3:2 POPC/Chol exhibits some positive intensity at the marker frequencies, suggesting that Chol has a limited ability to stabilize a functional nAChR.

The effect of lipid composition on nAChR conformational equilibria was examined further by photolabeling with the conformationally sensitive probe $[^{125}\text{I}]\text{TID}$ (14, 26–28). In 3:1:1 POPC/POPA/Chol membranes (as well as native *Torpedo* membranes), $[^{125}\text{I}]\text{TID}$ photoincorporates into each nAChR subunit with ~ 4 -fold greater incorporation into the γ -subunit relative to each of the other subunits (Fig. 4). Addition of agonist and subsequent desensitization of the nAChR results in a dramatic reduction in the extent of $[^{125}\text{I}]\text{TID}$ incorporation into each subunit. The ratio of the extent of $[^{125}\text{I}]\text{TID}$ incorporation into the γ - and α -subunits is a particularly sensitive indicator of the conformational state of the nAChR with a high ratio indicative

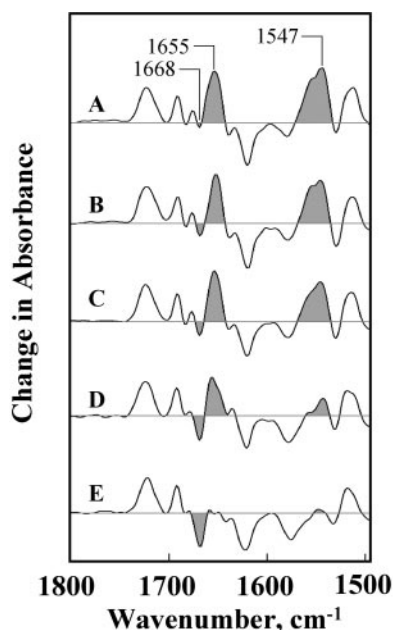


FIG. 3. A selected region of Carb difference spectra recorded from the nAChR reconstituted into membranes composed of 3:1:1 POPC/POPA/Chol (A), 3:2 POPC/DOPA (B), 3:2 POPC/POPA (C), 3:2 POPC/Chol (D), and POPC (E). Positive intensity near 1663, 1655, and 1547 cm⁻¹ is associated with the resting to desensitized conformational change. Note that a change in intensity near 1663 cm⁻¹ is most easily visualized by a change in overlapping negative band intensity near 1668 cm⁻¹ (22). Positive intensity at each of these frequencies in traces A–C indicates that the nAChR in 3:1:1 POPC/POPA/Chol, 3:2 POPC/DOPA, and 3:2 POPC/POPA undergoes desensitization upon Carb binding. The absence of positive intensity in trace E indicates that the nAChR in POPC cannot respond to Carb binding and is likely stabilized in a desensitized state. The nAChR in 3:2 POPC/Chol (trace D) exhibits some intensity at each frequency, suggesting a limited ability to respond to Carb binding. A small proportion of the nAChRs may be stabilized in a resting-like state in 3:2 POPC/Chol. Both the horizontal line and the shading are included in each spectrum for a visual reference.

of a resting conformation ($\gamma/\alpha = 4.11$; Table I) and a ratio equal to or less than unity indicative of a desensitized conformation (14, 27).

A similar pattern of [¹²⁵I]TID labeling in the absence and presence of agonist (Carb) is observed for the nAChR reconstituted into 3:2 POPC/POPA and 3:2 POPC/DOPA membranes (Fig. 4) showing, in agreement with the FTIR data, that both membranes stabilize a functional receptor that is capable of undergoing agonist-induced conformational transitions. In contrast, the nAChR in POPC exhibits much lower levels of [¹²⁵I]TID incorporation at equivalent exposures (see legend for Fig. 4) to that observed in the 3:1:1 POPC/POPA/Chol membranes in the absence of agonist. The lack of any change in the extent of [¹²⁵I]TID incorporation upon addition of Carb confirms that the nAChR in POPC is unable to undergo agonist-induced conformational transitions. Both the low overall extent of labeling and the γ/α ratio in the absence and presence of Carb (0.9 and 0.84, respectively; Table I) show that the nAChR in POPC is stabilized predominantly in a desensitized-like state. In four of five reconstitutions, the labeling pattern of the nAChR in 3:2 POPC/Chol was suggestive of a desensitized nAChR. The latter result is in agreement with the limited ability of the nAChR to stabilize a resting-like state in the POPC/Chol membranes as detected by FTIR difference spectroscopy.

Physical Properties of the Reconstituted nAChR Membranes—Two regions of the FTIR spectra exhibit bands that provide qualitative insight into the physical properties of the

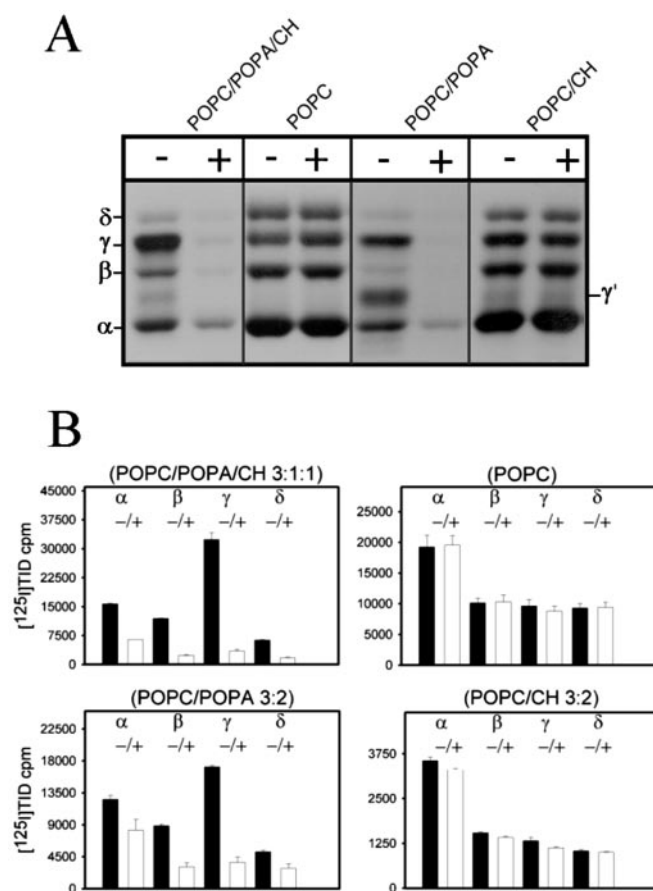


FIG. 4. Effects of Carb on the photoincorporation of [¹²⁵I]TID into the nAChRs reconstituted into defined lipids. Affinity-purified nAChR reconstituted into membranes composed of 3:1:1 POPC/POPA/Chol, 3:2 POPC/POPA, 3:2 POPC/DOPA (data not shown), 3:2 POPC/CH, and POPC was equilibrated 2 h with [¹²⁵I]TID (0.4 μM) in the absence (- lanes) and in the presence (+ lanes) of 400 μM Carb, irradiated at 365 nm for 7 min, and the polypeptides resolved by SDS-PAGE. A, corresponding autoradiographs of gels containing the [¹²⁵I]TID labeling experiments for each of the defined lipid environments. The positions of the nAChR subunits are indicated on the left. Note that the autoradiographs for the 3:2 POPC/Chol and POPC samples were exposed for much longer times (9 and 6 h, respectively) than the autoradiographs for the 3:1:1 POPC/POPA/Chol and 3:2 POPC/POPA samples (2 h each). B, for each [¹²⁵I]TID labeling experiment, individual nAChR subunit bands were excised from the dried gel and the amount of [¹²⁵I]TID photoincorporated into each subunit determined by γ counting (10 min of counting time). Shown are bar graphs of the amount of [¹²⁵I] cpm incorporated into each nAChR subunit in the absence or presence of carbamylcholine (-/+) and presented as the average of triplicate determinations.

reconstituted membranes and allow us to make preliminary conclusions regarding the existence of a correlation between the physical properties of the membranes and their ability to stabilize a functional nAChR. The lipid ester C=O stretching vibration between 1760 and 1700 cm⁻¹ is composed of two bands centered near 1740 and 1730 cm⁻¹ (Fig. 5), resulting from non-hydrogen-bonded and hydrogen-bonded lipid ester carbonyls, respectively (29, 33). Spectra recorded from reconstituted nAChR membranes composed of 3:1:1 POPC/POPA/Chol, 3:2 POPC/POPA, and 3:2 POPC/DOPA membranes all give rise to similar lipid ester carbonyl stretching band shapes with a relatively large proportion of ester carbonyls in the non-hydrogen-bonded state (Fig. 5, right panel). The large proportion of non-hydrogen-bonded lipid ester carbonyls in each case suggests a low degree of water penetration into the interfacial region of the lipid bilayer and thus a high density of head group packing. In contrast, the nAChR in POPC exhibits a

TABLE I

Ratio of [^{125}I]TID photoincorporation into the nAChR γ - and α -subunit in the absence and in the presence of Carb

nAChRs reconstituted into lipid vesicles of defined composition were labelled with [^{125}I]TID in the absence (- Carb) or presence (+ Carb) of 400 μM Carb. Following electrophoresis the nAChR subunit bands were excised and the amount of [^{125}I] cpm determined by γ counting (10 min of counting time). The γ/α is the ratio of [^{125}I] cpm (*i.e.* [^{125}I]TID) incorporation into the γ -subunit relative to the α -subunit. The values in parentheses are the standard error ($n = 3$).

Lipid composition of reconstituted nAChR vesicles	γ/α ratio	
	- Carb	+ Carb
POPC/POPA/Chol 3:1:1	4.11 (0.16)	1.06 (0.09)
POPC/DOPA 3:2	2.10 (0.04)	0.67 (0.01)
POPC/POPA 3:2	2.72 (0.11)	0.90 (0.08)
POPC/Chol 3:2	0.74 (0.02)	0.68 (0.01)
POPC	0.90 (0.12)	0.84 (0.07)

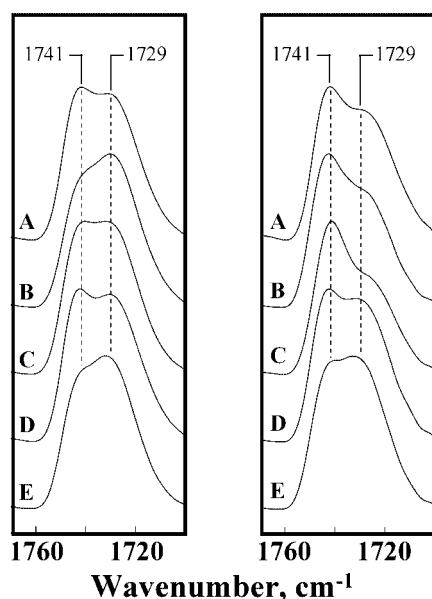


FIG. 5. The carbonyl stretching region in deconvoluted spectra of lipid membranes composed of 3:1:1 POPC/POPA/Chol (A), 3:2 POPC/DOPA (B), 3:2 POPC/POPA (C), 3:2 POPC/Chol (D), and POPC (E). The left panel shows the deconvoluted carbonyl stretching band in spectra of pure lipid membranes. The right panel shows the deconvoluted carbonyl stretching band in spectra of the same lipids, but in membranes with the nAChR at the lipid:protein ratios specified in Table II. All spectra were recorded at 22.5 °C. Note that the spectrum of the nAChR in 3:2 POPC/POPA was recorded just below the gel-to-liquid crystal phase transition, whereas all other spectra were recorded in the liquid crystal state. Transition from the gel to the liquid crystal phase leads to a slight reduction in the intensity of the 1741 cm^{-1} band caused by non-hydrogen-bonded lipid ester carbonyls as shown in Fig. 8. All traces have been base line-corrected between 1770 and 1700 cm^{-1} .

much larger proportion of hydrogen-bonded lipid ester carbonyls consistent with a greater degree of water penetration into the bilayer and a less ordered membrane. The nAChR in 3:2 POPC/Chol exhibits an intermediate pattern consistent with the relatively weak ability of Chol to stabilize a functional nAChR, although interpretation of the data is likely complicated because the hydroxyl of Chol may hydrogen bond with lipid ester carbonyls altering the shape of the stretching band. Note that within the limits observed in this study, variations in lipid-to-protein ratio had no effect on the interpretation of the data in terms of the relative proportion of hydrogen-bonded versus non-hydrogen-bonded lipid ester carbonyls in the different membranes.

Surprisingly, we observed substantial differences in the lipid carbonyl ester stretching band shapes in membranes either with or without the nAChR. In all cases, incorporation of the

nAChR into the lipid bilayers leads to an increase in the percentage of non-hydrogen-bonded lipid ester carbonyls, suggesting that the nAChR induces an ordering of the membrane. Although this is not surprising given the size of the nAChR (~270,000 Da), it is significant that the increase in ordering observed upon incorporation of the nAChR into the lipid bilayers is much greater for those membranes that contain the anionic lipids DOPA and POPA. For example, the 3:2 POPC/DOPA and POPC lipid membranes lacking the nAChR both exhibit lipid ester carbonyl stretching vibrations at 22.5 °C, indicative of a similar high degree of water penetration into the lipid bilayer, suggesting a similar degree of membrane order (Fig. 5). In contrast, the 3:2 POPC/DOPA membranes appear to be substantially more ordered than the POPC membranes, in the presence of the nAChR. This result suggests that the nAChR selectively influences the physical properties of phosphatidic acid-containing lipid bilayers.

A similar conclusion was reached upon examination of the gel-to-liquid crystal phase transition temperatures of the various membranes measured in the presence and absence of the nAChR. Gel-to-liquid crystal phase transition temperatures were defined by following the symmetric C-H stretching frequencies of the lipid acyl chains, which decreases by roughly 2 cm^{-1} upon transition from the liquid crystal to the gel state. Cooling curves for the different lipid membranes in the absence of the nAChR (*open circles*) vary substantially depending on the degree of unsaturation of the lipid bilayers (Fig. 6). As expected, high levels of Chol abolish the phase transition and order lipids in the liquid crystal state. Incorporation of the nAChR (*closed circles*) into membranes composed of POPC leads to only a slight broadening of the gel-to-liquid crystal phase transition and very slight shift to higher temperatures. In contrast, incorporation of the nAChR into each membrane that contains either POPA or DOPA leads to a marked increase in the gel-to-liquid crystal phase transition temperature. For example, the gel-to-liquid crystal phase transition temperature for the 3:2 POPC/DOPA membranes shifts by roughly 15 °C upon incorporation of the nAChR. This result supports further our conclusion that the nAChR selectively modulates the physical properties of POPC membranes containing the anionic lipids POPA or DOPA.

Does Incorporation of the nAChR into 3:2 POPC/POPA Membranes Lead to a Phase Separation?—The ability of the nAChR to selectively influence the physical properties of POPC membranes containing either DOPA or POPA may reflect a direct interaction between the nAChR and anionic lipids. Such an interaction could lead to a phase separation between POPC and either POPA or DOPA in the presence of the nAChR. Given the roughly 30 °C difference in phase transitions of pure POPC and POPA membranes (Table II), a phase separation of the two lipids in the reconstituted 3:2 POPC/POPA might lead to the formation of lipid environments with distinct phase transition temperatures. Although the cooling curves presented in Fig. 6 show no evidence for two distinct transition temperatures, we tested this possibility further by recording cooling curves for the nAChR reconstituted into 3:2 POPC/POPA membranes where POPA was perdeuterated along the palmitoyl chain. Incorporation of ^2H shifts the symmetric C-H stretching frequency of the acyl chain by roughly 800 cm^{-1} , allowing for the phase transition of POPA in the reconstituted 3:2 POPC/POPA membranes to be monitored individually. As shown in Fig. 7, both POPA and POPC in the reconstituted 3:2 POPC/POPA membranes undergo the gel-to-liquid crystal phase transition at roughly the same temperature, suggesting that the reconstituted 3:2 POPC/POPA membranes are a homogeneous mixture. Although not definitive, this result argues against a

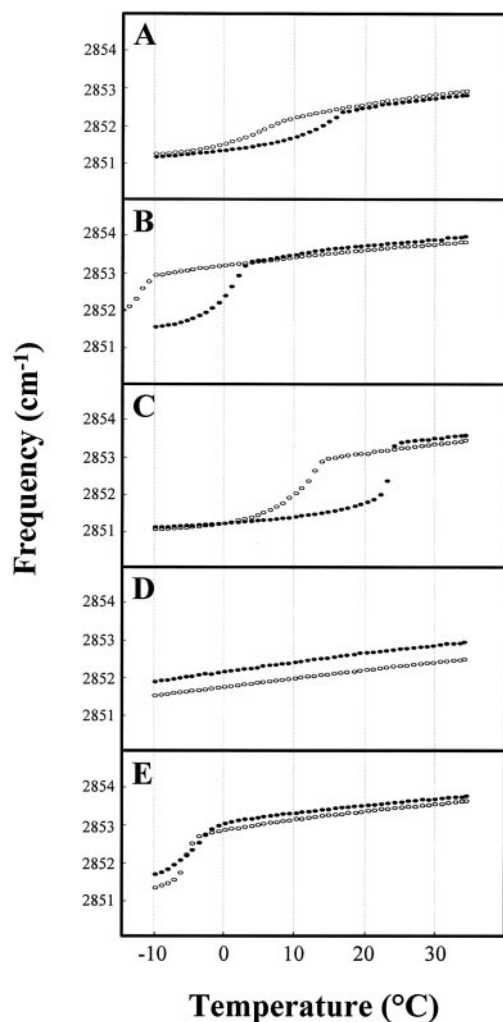


FIG. 6. Temperature dependence of the C-H symmetric stretching frequencies observed in spectra of membranes composed of 3:1:1 POPC/POPA/Chol (A), 3:2 POPC/DOPA (B), 3:2 POPC/POPA (C), 3:2 POPC/Chol (D), and POPC (E) either with (filled circles) or without (clear circles) the nAChR. For more details, see "Experimental Procedures."

phase separation of zwitterionic and anionic lipids in the presence of the nAChR.

Effect of Gel-to-Liquid Crystal Phase Transition on the Ability of the nAChR to Undergo Agonist-induced Conformational Change—The nAChR-reconstituted 3:2 POPC/POPA membranes have a phase transition temperature that is close to room temperature (Table II). Both the difference spectra and [¹²⁵I]TID labeling experiments presented in Figs. 3 and 4 were performed at 22.5 °C, suggesting that the nAChR may be functional in a membrane that contains a substantial proportion of gel state lipids. To examine in more detail the effects of the gel state on the functional capabilities of the nAChR, Carb difference spectra were recorded above (30 °C) and below (15 °C) the gel-to-liquid crystal phase transition (Fig. 8). [¹²⁵I]TID labeling was also monitored in the gel at 4 °C (data not shown). Both techniques suggest that the nAChR in a gel state membrane retains the ability to undergo agonist-induced conformational change. Analysis of the lipid C=O stretching vibration confirms the increased order of the POPC/POPA membranes in the gel state (Fig. 8). The ability of the nAChR to undergo agonist-induced conformational transitions appears to be unaffected by highly rigid gel state lipid bilayers.

DISCUSSION

A long term goal of this work is to understand the mechanisms underlying the relative abilities of reconstituted nAChR membranes composed of either POPC alone or POPC with either POPA and/or Chol to stabilize the nAChR in a functional conformation. In this report, we focused on the link between membrane fluidity and nAChR conformational equilibria proposed in Fig. 1. Although the results generally support the existence of such a link, the data illustrate the complexity of the physical properties of even these simple reconstituted nAChR membranes and thus the need for more comprehensive ²H NMR/molecular modeling studies to fully test our working model. The data also reveal several unanticipated features regarding the interactions that occur between the nAChR and lipid bilayers.

FTIR difference spectroscopy and [¹²⁵I]TID labeling both indicate, in agreement with previous studies of similar membranes (3, 5, 14, 30), that the nAChR reconstituted into membranes composed of POPC alone is stabilized in a desensitized-like state that does not undergo agonist-induced conformational change. The reconstituted POPC membranes have relatively low gel-to-liquid crystal phase transition temperatures and exhibit lipid ester carbonyl stretching band shapes indicative of a high degree of water penetration into the lipid head group region of the bilayer and thus a low lateral lipid packing density. Both are consistent with a relatively disordered or fluid membrane at room temperature.

In contrast, the nAChR in 3:2 POPC/POPA and 3:2 POPC/DOPA membranes is stabilized in a resting-like state that is capable of undergoing agonist-induced desensitization. Although the gel-to-liquid crystal phase transition temperatures of the nAChR in 3:2 POPC/POPA and 3:2 POPC/DOPA differ, they are both higher than the phase transition temperature of the reconstituted POPC membranes. The lipid carbonyl ester stretching band shapes suggest a high degree of lateral packing density in the POPA- and DOPA-containing lipid bilayers reconstituted with the nAChR and thus a higher degree of membrane order. In addition, the presence of phosphatidic acid in phosphatidylcholine membranes appears to slow down nAChR internal motions as monitored by a slowing of exchange kinetics for all nAChR peptide hydrogens (15). Collectively, these results are consistent with the hypothesis that relatively ordered membranes stabilize the receptor in a resting-like state whereas relatively fluid membranes favor a desensitized conformation.

The correlation between fluidity and function is less clear for the reconstituted POPC membranes containing Chol. The nAChR in 3:2 POPC/Chol appears to be predominantly desensitized, although the FTIR data suggest a limited ability to stabilize receptors in a resting-like state. The lipid ester carbonyl stretching band shape of the reconstituted POPC/Chol membranes is intermediate between that observed in the presence and absence of either POPA or DOPA, consistent with a limited ability to stabilize a functional nAChR. On the other hand, 40 mol% Chol in the POPC membranes abolishes the gel-to-liquid crystal phase transition and, based on the relatively low methylene C-H stretching frequencies, leads to a bilayer with acyl chains that appear to be more ordered in the liquid crystal phase than the acyl chains in the reconstituted 3:2 POPC/DOPA and 3:2 POPC/POPA membranes. According to our working model, the latter requires that the 3:2 POPC/Chol membrane should favor the resting state. It is possible, however, that the POPC membranes containing Chol are ordered in a manner that is different from the ordering that occurs in POPC membranes containing either POPA or DOPA.

Note that other studies have reported a greater ability of

TABLE II
Gel-to-liquid crystal phase transition temperatures of the lipid membranes measured in the presence and absence of the nAChR

Membrane lipid composition	Lipid:Protein (mole:mole)	T_m of pure lipid	T_m of lipid + nAChR	Change in T_m with nAChR
		°C	°C	°C
POPC/POPA/Chol, 3:1:1	330:1	3.8	12.6	+8.8
POPC/DOPA, 3:2	184:1	-12.9	0.5	+13.4
POPC/POPA, 3:2	150:1	10.7	23.7	+13.0
POPC/Chol, 3:2	175:1	ND ^a	ND	ND
POPC	147:1	-6.1	-5.1	+1.0
POPA	ND	27.0	ND	ND

^a ND, not determined.

FIG. 7. Temperature dependence of the C-H (mainly POPC; closed circles, left axis) and C-²H (POPA; open triangles, right axis) symmetric stretching frequencies in spectra of the nAChR reconstituted into membranes composed of 3:2 POPC/POPA with deuterium labels along the palmitoyl chain of POPA. Note that the sample contains both deuterium-labeled and non-deuterium-labeled POPA. The relatively weak C-²H signal also overlapped with the vibration of ²H₂O, leading to a relatively poor signal-to-noise.

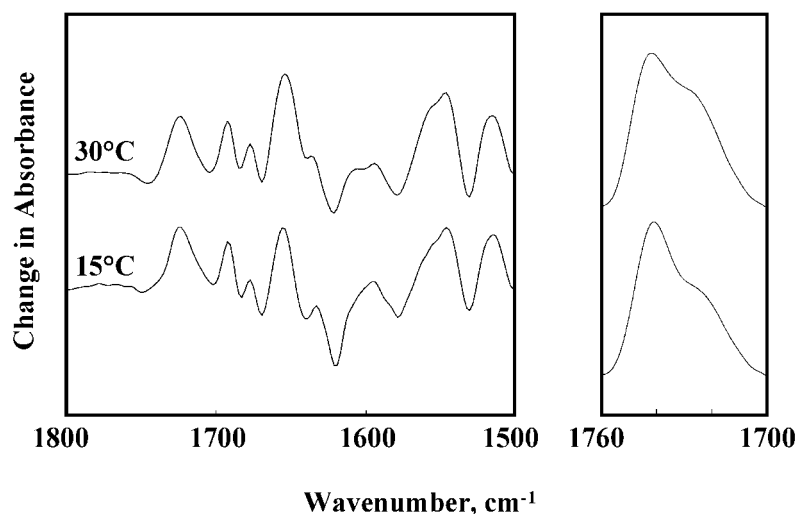
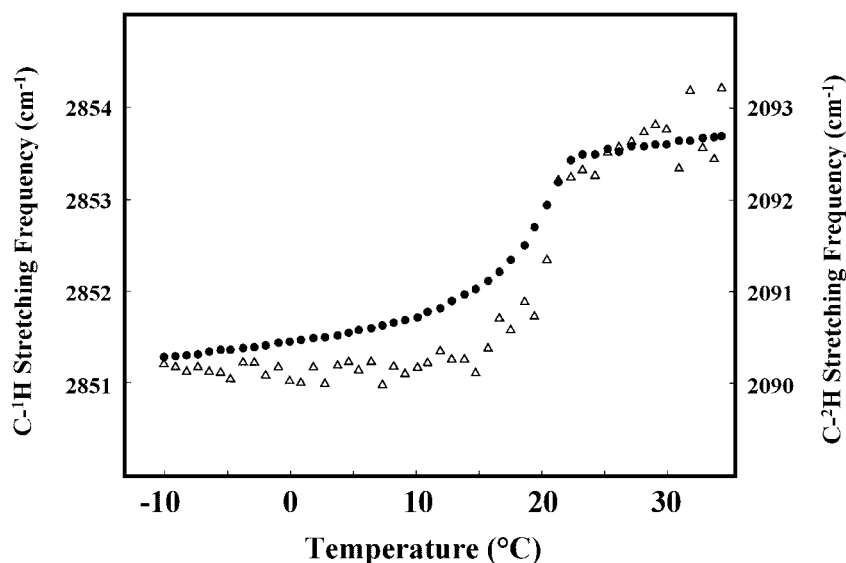


FIG. 8. The left panel is a selected region of Carb difference spectra recorded from the nAChR reconstituted into membranes composed of 3:2 POPC/POPA both above (30 °C) and below (15 °C) the liquid crystal phase transition temperature. The right panel shows the lipid ester carbonyl stretching band of the nAChR membranes at the corresponding temperatures. The photoincorporation of [¹²⁵I]TID into the nAChR in 3:2 POPC/POPA at 4 °C was indistinguishable from that observed at room temperature both in the presence and absence of Carb (data not shown).

Chol in either EPC or DOPC membranes to support a functional nAChR that undergoes agonist-induced conformational change (5, 30). In EPC and DOPC, 40 and 30 mol% Chol, respectively, are optimal for stabilizing the nAChR in a conformation that is capable of undergoing agonist-induced conformational change. The ability of the nAChR to respond to agonist binding in either EPC or DOPC, however, is diminished at both higher and lower levels of Chol (5). Levels of Chol different from those used here in the POPC membranes may be more effective at stabilizing a resting-like state. In addition, it is possible that DOPC or EPC membranes containing Chol are more effective in creating an environment suitable for the nAChR than POPC/Chol mixtures.

Our data also show, in agreement with several other functional studies (3, 13, 14, 30), that the nAChR in 3:1:1 POPC/POPA/Chol membranes is stabilized in a fully functional state. This result is surprising given our previous work (5), which suggests that either 20% Chol or 20% POPA alone in a POPC membrane is likely to have a very limited ability to stabilize the nAChR in a functional conformation. It appears that Chol and POPA act synergistically in POPC membranes to modulate nAChR conformational equilibria. Unfortunately, the qualitative analysis of membrane physical properties reported here is inadequate to fully understand the unique physical properties of the 3:1:1 POPC/POPA/Chol membrane and why the membrane is particularly effective at stabilizing a functional

nAChR. An understanding of how this slightly more complex membrane stabilizes a functional nAChR is important, given that the physical environment of the 3:1:1 POPC/POPA/Chol membranes is likely more representative of that found in native membranes.

A surprising, but significant finding of this study is that the physical properties of the "functional" POPC membranes containing either DOPA or POPA are strongly influenced by the presence of the nAChR, whereas the physical properties of the "nonfunctional" pure POPC membranes are relatively unaffected. For all the phosphatidic acid-containing membranes, incorporation of the nAChR leads to a large increase in the gel-to-liquid crystal phase transition temperature as well as a large increase in the lateral packing density of the lipid bilayers, the latter reflected by a decrease in water penetration into the bilayer surface and thus a decrease in the number of hydrogen-bonded lipid ester carbonyls. Large effects of the nAChR upon the bulk physical properties of any lipid bilayer have not been reported previously. To the best of our knowledge, large changes in gel-to-liquid crystal phase transitions have not been reported upon incorporation of any integral membrane protein into a lipid bilayer. The FTIR data, although qualitative, also indicate that the physical properties of some nAChR membranes are not governed by the intrinsic properties of the lipids alone. The nAChR plays a substantial role in defining the physical characteristics of the reconstituted membranes.

The ability of the nAChR to selectively influence the physical properties of membranes containing POPA or DOPA is particularly intriguing, given that both lipids are uniquely effective at stabilizing the nAChR in a functional state. These results suggest the nAChR interacts in a unique fashion with POPA and DOPA-containing lipid bilayers. In the reconstituted 3:2 POPC/POPA membranes, this interaction does not likely lead to or result from a lateral phase separation of POPC and POPA because the membranes behave as a homogeneous mixture in terms of the gel-to-liquid crystal phase transition. The ability of the nAChR to modulate the physical properties of the bilayers could stem from hydrophobic mismatching between the nAChR and the fatty acyl chains of the POPC/POPA or POPC/DOPA membranes. A hydrophobic mismatch could lead to large shifts in the gel-to-liquid crystal phase transition temperature upon incorporation of the nAChR (34). Regardless of the precise mechanism, it seems plausible that the unique coupling between the nAChR and the physical properties of the phosphatidic acid-containing membrane may play an important role in how phosphatidic acid modulates nAChR function.

Work by others has also suggested a unique role for anionic lipids in modulating nAChR function, although the additional presence of Chol is usually required to stabilize a fully functional nAChR (3, 4, 10, 11). We cannot detect the changes in secondary structure of the nAChR in the presence of either POPA or DOPA that have been detected by others (4, 10, 11) nor do we detect changes in the pK_a values of the phosphate stretching vibrations of either POPA or DOPA upon incorporation of the nAChR (data not shown). We are currently trying to define the structural features of phosphatidic acid that make it such an effective determinant of nAChR function. For example,

the importance of the negative charge and/or the small head-group of phosphatidic acid is being tested by assessing the structural and functional characteristics of the nAChR in POPC membranes composed of either 1-palmitoyl-2-oleoyl phosphatidyl serine or 1-palmitoyl-2-oleoyl phosphatidyl ethanolamine.

We have characterized the structure and conformational states of the nAChR stabilized in POPC, 3:2 POPC/POPA, 3:2 POPC/Chol, and 3:1:1 POPC/POPA/Chol membranes. Each lipid used in these membranes is available in a deuterated form. We are now in a position to rigorously analyze the physical properties of the reconstituted membranes using ^2H NMR spectroscopy. These future studies should provide important insight into the mechanisms by which membrane lipids influence integral membrane protein function, in general.

REFERENCES

- 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
- Barrantes, F. J. (1989) *Crit. Rev. Biochem. Mol. Biol.* **24**, 437–478
- Fong, T. M., and McNamee, M. G. (1986) *Biochemistry* **25**, 830–840
- Fong, T. M., and McNamee, M. G. (1987) *Biochemistry* **26**, 3871–3880
- Baenziger, J. E., Morris, M.-L., Darsaut, T. E., and Ryan, S. E. (2000) *J. Biol. Chem.* **275**, 777–784
- Marsh, D., and Barrantes, F. J. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 4329–4333
- Ellena, J. F., Blazing, M. A., and McNamee, M. G. (1983) *Biochemistry* **22**, 5523–5535
- Criado, M., Eibl, H., and Barrantes, F. J. (1982) *Biochemistry* **21**, 3622–3629
- Butler, D. H., and McNamee, M. G. (1993) *Biochim. Biophys. Acta* **1150**, 17–24
- Bhushan, A., and McNamee, M. G. (1993) *Biophys. J.* **64**, 716–723
- Fernandez-Ballester, G., Castresana, J., Fernandez, A. M., Arrondo, J.-L. R., Ferragut, J. A., and Gonzalez-Ros, J. M. (1994) *Biochemistry* **33**, 4065–4071
- Méthot, N., Demers, C. N., and Baenziger, J. E. (1995) *Biochemistry* **34**, 15142–15149
- Ryan, S. E., Demers, C. N., Chew, J. P., and Baenziger, J. E. (1996) *J. Biol. Chem.* **271**, 24590–24597
- McCarthy, M. P., and Moore, M. A. (1992) *J. Biol. Chem.* **267**, 7655–7663
- Baenziger, J. E., Darsaut, T. E., and Morris, M.-L. (1999) *Biochemistry* **38**, 4905–4911
- Sunshine, C., and McNamee, M. G. (1994) *Biochim. Biophys. Acta* **1191**, 59–64
- Criado, M., Eibl, H., and Barrantes, F. J. (1984) *J. Biol. Chem.* **259**, 9188–9198
- Auger, M., Carrier, D., Smith, I. C. P., and Jarrell, H. C. (1990) *J. Am. Chem. Soc.* **112**, 1373–1381
- Baenziger, J. E., Jarrell, H. C., and Smith, I. C. P. (1992) *Biochemistry* **31**, 3377–3385
- Goormaghtigh, E., Cabiaux, V., and Ruyschaert, J. M. (1990) *Eur. J. Biochem.* **193**, 409–420
- Baenziger, J. E., Miller, K. W., and Rothschild, K. J. (1992) *Biophys. J.* **61**, 983–992
- Ryan, S. E., and Baenziger, J. E. (1999) *Mol. Pharmacol.* **55**, 348–355
- Méthot, N., McCarthy, M. P., and Baenziger, J. E. (1994) *Biochemistry* **33**, 7709–7717
- Baenziger, J. E., and Méthot, N. (1995) *J. Biol. Chem.* **270**, 29129–29137
- Méthot, N., and Baenziger, J. E. (1998) *Biochemistry* **37**, 14815–14822
- White, B. H., and Cohen, J. B. (1992) *J. Biol. Chem.* **267**, 15770–15783
- Blanton, M. P., McCarty, E. A., and Gallagher, M. J. (2000) *J. Biol. Chem.* **275**, 3469–3478
- Chiara, D. C., Kloczewiak, M. A., Addona, G. H., Yu, J.-A., Cohen, J. B., and Miller, K. W. (2001) *Biochemistry* **40**, 296–304
- Hubner, W., and Mantsch, H. H. (1991) *Biophys. J.* **59**, 1261–1272
- Rankin, S. E., Addona, G. H., Kloczewiak, M. A., Bugge, B., and Miller, K. W. (1997) *Biophys. J.* **73**, 2446–2455
- Reid, S. E., Moffatt, D. J., and Baenziger, J. E. (1996) *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **52**, 1347–1356
- Seelig, J., and Waespe-Sarcevic, N. (1978) *Biochemistry* **17**, 3310–3315
- Blume, A., Hubner, W., and Messner, G. (1988) *Biochemistry* **27**, 8239–8249
- Gil, T., Ipsen, J. H., Mouritsen, O. G., Sabra, M. C., Sperotto, M. M., and Zuckermann, M. J. (1998) *Biochim. Biophys. Acta* **1376**, 245–266
- Mendelsohn, R., and Mantsch, H. H. (1986) in *Progress in Protein-Lipid Interactions 2* (Watts, A., and De Pont, J. J., eds) pp. 103–146, Elsevier Science Publishers, B.V., Amsterdam