Phosphatidic Acid and Phosphatidylserine Have Distinct Structural and Functional Interactions with the Nicotinic Acetylcholine Receptor*

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Bilayers containing phosphatidylcholine (PC) and the anionic lipid phosphatidic acid (PA) are particularly effective at stabilizing the nicotinic acetylcholine receptor (nAChR) in a functional conformation that undergoes agonist-induced conformational change. The physical properties of PC membranes containing PA are also substantially altered upon incorporation of the nAChR.

To test whether or not the negative charge of PA is responsible for this “bi-directional coupling,” the nAChR was reconstituted into membranes composed of PC with varying levels of the net negatively charged lipid phosphatidylserine (PS). In contrast to PA, increasing levels of PS in PC membranes do not stabilize an increasing proportion of nAChRs in a functional resting conformation, nor do they slow nAChR peptide hydrogen exchange kinetics. Incorporation of the nAChR had little effect on the physical properties of the PC/PS membranes, as monitored by the gel-to-liquid crystal phase transition temperatures of the bilayers. These results show that a net negative charge alone is not sufficient to account for the unique interactions that occur between the nAChR and PC/PS membranes. Incorporation of the receptor into PC/PS membranes, however, did lead to an altered head group conformation of PS possibly by recruiting divalent cations to the membrane surface. The results show that the nAChR has complex and unique interactions with both PA and PS. The interactions between the nAChR and PS may be bridged by divalent cations, such as calcium.

The ability of the nicotinic acetylcholine receptor (nAChR) to flux cations across the post-synaptic membrane in response to agonist binding is influenced by the composition of its surrounding lipid environment (1, 2). Although detailed insight into the underlying mechanisms has not yet been achieved, a current model proposes that lipid bilayers modulate nAChR function by altering the natural equilibrium that exists between “functional” resting and “nonfunctional” desensitized conformational states (3). In native post-synaptic membranes, this equilibrium strongly favors the resting conformation, allowing the nAChR to flux cations in response to agonist binding. The equilibrium is maintained in favor of the resting conformation in reconstituted bilayers composed of phosphatidylcholine (PC), an anionic lipid, such as phosphatidic acid (PA), and a neutral lipid, such as cholesterol (Chol) (3–5). In contrast, the equilibrium shifts in favor of a desensitized or a desensitized-like state, leading to channel inactivation in pure PC membranes (4–8). The effects of neutral and anionic lipids on nAChR conformational equilibria are not dependent upon a global change in secondary structure (9). The presence of either or both lipids in a reconstituted PC membrane does slow nAChR peptide hydrogen exchange kinetics, suggesting a decrease in the rates and/or amplitudes of internal nAChR motions (10).

Additional experiments have expanded on this simple model of lipid action, showing that the addition of either PA or Chol alone to a PC membrane is sufficient to shift the conformational equilibrium toward the resting state (3, 6). The degree of the conformational shift depends on both the type and amount of lipid added, with PA being particularly effective at stabilizing a resting conformation (3, 7, 11). As increasing amounts of either Chol or PA in a PC membrane could lower bilayer fluidity, we suggested that both lipids may shift the conformational equilibrium in favor of the resting state by decreasing the fluidity of the membrane surrounding the nAChR (3, 11). Any links that exist between membrane physical properties and nAChR function, however, are likely complex (11).

A recent study also showed that incorporation of the nAChR into PC membranes containing PA leads to a substantial increase in both the gel-to-liquid crystal phase transition temperature and the lateral packing density of the lipids. In contrast, incorporation of the nAChR into membranes composed of either PC or PC/Chol has little effect (11, 12). The ability of the nAChR to selectively modulate the physical properties of PC membranes containing PA is of particular interest given that PC/PA membranes are especially effective at stabilizing a functional nAChR. These results suggest that there is a bi-directional “coupling” between the nAChR and PA-containing lipid bilayers in that the lipids modulate nAChR structure/function, whereas in return the nAChR modulates lipid structure and possibly function. This bi-directional coupling may underlie the unique mechanism by which PA influences nAChR conformational equilibria and thus stabilizes a functional nAChR.

To understand the mechanism(s) by which PA modulates nAChR conformational equilibria, it is first necessary to define...
the structural features of PA that allow it to interact with the nAChR in a unique fashion. To test whether or not a net negative charge is sufficient to account for these interactions, we have reconstituted the nAChR into PC membranes containing varying levels of the lipid phosphatidylserine (PS). PS has a net negative charge and is abundant in Torpedo post-synaptic membranes. Surprisingly, our results show that despite its net negative charge, increasing (POPA), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (POP) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (P-OPS) were from Avanti Polar lipids, Inc. (Alabaster, AL), and both cholesterol and carbamylcholine chloride (Carb) were from Sigma.

EXPERIMENTAL PROCEDURES

Materials—Frozen Torpedo californica electroplax tissue was obtained from either Marinus (Long Beach, CA) or Aquatic Research Consultants (San Pedro, CA). 2-Aminoethyl-3-aminopropyl-trichlorosilane from DuPont NEN Research Products (Boston, MA), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (P-OPS) were from Avanti Polar lipids, Inc. (Alabaster, AL), and both cholesterol and carbamylcholine chloride (Carb) were from Sigma.

FTIR Spectroscopy—FTIR spectra of the nAChR affinity-purified and reconstituted into membranes composed of POPC, POPC/POPS 3:2 (mol/mol), POPC/POPA 3:2 (mol/mol), and POPC/POPS/Chol 3:1:1 (mol/mol/mol) each exhibit four main features in the 1800–1400 cm⁻¹ region (Fig. 1). These four features are mainly attributable to the protein amide I (1700–1600 cm⁻¹), amide II (1520–1580 cm⁻¹), and amide III (1500–1400 cm⁻¹) vibrations, as well as to the ester carbonyl stretching vibrations of the membrane phospholipid (1750–1700 cm⁻¹). The shapes and relative intensities of the three protein bands reflect structural features of the nAChR in each membrane, whereas the shape of the lipid ester carbonyl vibration reflects the structure of the surrounding lipid environment (see below). Subtle differences are observed in each of the vibrational bands between spectra recorded from the nAChR reconstituted into POPC/POPS 3:2 and POPC/POPA 3:2 membranes (Fig. 1). These variations are typical of the variations observed between spectra recorded from the nAChR in each of the five lipid environments and provide insight into the mechanisms by which the nAChR couples its structure and function to the composition of its surrounding lipid bilayer.

nAChR Structure and Internal Dynamics—The amid I band between 1600 and 1700 cm⁻¹ is due predominantly to the peptide carbonyl (C=O) stretching vibration, and its shape is sensitive to hydrogen bonding and thus protein secondary structure. Deconvolution of the amid I band provides a clearer view of the frequencies and relative intensities of the underlying component vibrations and thus permits a qualitative assessment of the secondary structure of the nAChR in each lipid environment (Fig. 2, left panel) (15). In each case, the decon-
The deconvolved amide I band (left panel) and residual amide II band intensity (right panel) in FTIR spectra recorded from the nAChR reconstituted into 3:2 POPC/POPA (A), 3:1 POPC/POPC/Chol (B), 3:2 POPC/POPS (C), 3:1 POPC/POPC (D), and POPC (E) membranes. The amide I bands presented in the left panel were deconvolved between 1900 and 1300 cm\(^{-1}\) with a γ factor of 7 and a smoothing parameter of 70%. Amide II bands presented in the right panel are not deconvolved. All of the samples were exposed to \(^2\)H\(_2\)O buffer for 72 h at 4 °C prior to data acquisition, and a spectrum of \(^2\)H\(_2\)O buffer has been subtracted from each. All of the samples are hydrated with \(^2\)H\(_2\)O Torpedo ringer buffer. The spectra were recorded at 22.5 °C.

Deconvolved amide I bands exhibit a similar number of underlying components at similar relative intensities, including two major peaks of intensity near 1655 and 1636 cm\(^{-1}\) because of α-helical and β-sheet secondary structures, respectively (15). The overall similarities of the deconvolved amide I band shapes indicate that the nAChR has roughly the same mixed α-helix/β-sheet structure in each membrane environment.

The non-deconvolved amide I bands in spectra recorded from the nAChR in POPC and mixtures of POPC and POPS are slightly downshifted in frequency relative to the amide I bands in spectra recorded from the nAChR in either POPC/POPA 3:2 or POPC/POPS/Chol 3:1:1 membranes (Fig. 1). Deconvolution shows that this downshift in frequency is due to a subtle shift in intensity from 1655 cm\(^{-1}\) down to 1645 cm\(^{-1}\) (Fig. 2, left panel). Although the decrease in α-helical amide I component band intensity could reflect a minor decrease in α-helical structure, previous studies have shown that the α-helical vibration undergoes a shift in frequency from 1655 cm\(^{-1}\) in \(^2\)H\(_2\)O down to near 1645 cm\(^{-1}\) in \(^2\)H\(_2\)O (16, 17). After 72 h of exposure to \(^2\)H\(_2\)O, roughly 25% of the nAChR peptide hydrogens remain unexchanged for deuterium, although the extent of peptide hydrogen/deuterium exchange is sensitive to lipid environment and thus a greater proportion of unexchanged peptide hydrogens relative to the nAChR in POPC and POPC/POPS (3:1 and 3:2) membranes. A previous study with the nAChR showed that similar variations in the levels of peptide hydrogen/deuterium exchange lead to similar changes in nAChR amide I band shape (9). These results therefore suggest that the secondary structure of the nAChR is not influenced by the presence of POPA, POPS, or both POPA and Chol in a reconstituted POPC membrane. Instead, these lipids modulate the amplitude and/or rates of nAChR internal motions, resulting in differences in the extent of peptide 1H-2H exchange.

Note that the presence of either 25 or 40 mol % POPS in a reconstituted POPC membrane does not lead to a slowing of nAChR peptide hydrogen exchange kinetics and thus a slowing of nAChR internal dynamics (relative to the nAChR in POPC membranes) as seen in the presence of 40 mol % POPA. This shows that negative charge alone is not sufficient to account for the effects of POPA or POPS on nAChR structure. The data also support the hypothesis that there is a correlation between the internal dynamics of the nAChR in different membranes and the ability of the membrane to stabilize the nAChR in a functional state (see “Discussion”).

nAChR Conformational Equilibria—The effects of bilayer lipid composition on nAChR conformational equilibria were examined using FTIR difference spectroscopy (Fig. 3). The difference between spectra of the nAChR recorded in the presence and absence of the agonist carbamylcholine (referred to as a Carb difference spectrum) exhibits a complex pattern of positive and negative bands that reflect the vibrational changes in the nAChR that occur upon Carb binding and subsequent desensitization. Positive and negative difference bands in the 1640–1670 cm\(^{-1}\) (mainly peptide C=O stretching) and 1520–1580 cm\(^{-1}\) (mainly peptide N–H bending) regions reflect the structural changes in the polypeptide backbone that occur upon transition from the resting to the desensitized conformational state. The pattern of overlapping difference bands in both regions of spectra recorded from the nAChR in POPC/POPA 3:2 membranes is similar to that observed in difference spectra recorded from the nAChR in native and PC/PA/Chol 3:1:1 membranes (data not shown). This pattern is characteristic of a receptor that is stabilized in a resting conformation that undergoes desensitization upon Carb binding.

Intensity is lost in both the 1640–1670 and 1520–1580 cm\(^{-1}\) spectral regions of difference spectra recorded from the nAChR reconstituted into POPC and POPC/POPS 3:1 and 3:2 membranes (Fig. 3, spectra C–E). The resulting pattern of bands is similar to that observed in difference spectra recorded from the nAChR reconstituted into PC/PA/Chol 3:1:1 membranes, although the nAChR is maintained in a desensitized conformation in the presence of a desensitizing local anesthetic, such as...
dibucaine. The pattern of bands observed in difference spectra recorded from the nAChR in either POPC alone or mixtures of POPC and POPS indicates that the nAChR cannot undergo the resting to desensitized conformational transition upon Carb binding. The spectra suggest that the nAChR is stabilized in a desensitized or desensitized-like conformation (see “Discussion” in Ref. 3). Based on the FTIR data, it is clear that the presence of POPS alone in a POPC membrane is not sufficient to shift the nAChR conformational equilibrium toward the resting state.

In contrast, Carb difference spectra recorded from the nAChR in POPC/POPS/Chol 3:1:1 membranes exhibit a pattern of positive and negative vibrations that is intermediate between that observed in POPC/POPS and POPC/POPA 3:2 membranes. The spectra suggest that mixtures of POPC/POPS and Chol are somewhat effective at stabilizing the nAChR in a resting conformation, although the degree of the conformational shift toward the resting state is not as great as observed in POPC/POPA 3:2 membranes.

Physical Properties of the Reconstituted nAChR Membranes—The effects of nAChR on the physical properties of the reconstituted lipid bilayers was first examined by comparing the gel-to-liquid crystal phase transition temperatures of the various POPC/POPS membranes in the presence and absence of the nAChR (Fig. 4). The methylene symmetric C–H stretching frequencies of the fatty acyl chains decreases by roughly 2 cm\(^{-1}\) upon transition from the liquid crystal to the gel state and can thus be used to monitor the gel-to-liquid crystal phase transition temperatures of the reconstituted bilayers (18). Cooling curves for the different lipid membranes in the absence of the nAChR (open circles) vary depending on the lipid composition (Fig. 4). As shown previously, incorporation of the nAChR (closed circles) into membranes composed of POPC (Fig. 4E) leads to a slight broadening of the gel-to-liquid crystal phase transition and very slight shift to higher temperatures, whereas incorporation of the nAChR into the 3:2 POPC/POPA
membrane (Fig. 4A) leads to a 15 °C increase in the gel-to-liquid crystal phase transition temperature (11) (Table I). In the POPC/POPS:Chol 3:3:1 and 3:2:2 membranes (Fig. 4, D and C), there is no observed shift in the phase transition temperature, but the difference in frequency between the gel and liquid crystalline phases is reduced upon nAChR incorporation, possibly because of the presence of calcium (19) (see below). Furthermore, the degree to which this difference in vibrational frequency between the liquid crystalline and gel states is reduced appears to be dependent on the amount of POPS in each membrane. The C–H stretching vibrations of the lipid acyl chains in the liquid crystalline phase of POPC/POPS/Chol 3:1:1 (Fig. 4B) also vibrate at a slightly lower frequency in the presence of the nAChR, essentially eliminating the gel-to-liquid crystal phase transition. Note that variations in the lipid to protein ratio cannot account for any of the variations in gel-to-liquid crystal phase transition (Table I).

The shape of the lipid ester C=O stretching vibration between 1760 and 1700 cm⁻¹ is also a qualitative monitor of bilayer physical properties. The band is composed of two components centered near 1741 and 1729 cm⁻¹ (Fig. 5) because of non-hydrogen-bonded and hydrogen-bonded lipid ester carbonyls, respectively (20). The relative proportion of these two vibrations is a qualitative indicator of the penetration of water into the bilayer interfacial region. A relatively large proportion of non-hydrogen-bonded lipid ester carbonyls near 1741 cm⁻¹, suggesting a low degree of water penetration into the interfacial region of the lipid bilayer and thus a high lateral density of lipid head group packing. Incorporation of the nAChR into POPC/POPS membranes leads to a large increase in the proportion of non-hydrogen-bonded lipid ester carbonyls, suggesting a substantial ordering of the POPC/POPA membrane. This observation is consistent with the effects of the nAChR on the gel-to-liquid crystal phase transition temperature of the nAChR.

Reconstitution of the nAChR into POPC has a minimal effect on the relative proportion of the two lipid ester carbonyl vibrations, suggesting minimal effects on the packing of the bilayer.

Surprisingly, incorporation of the nAChR into membranes composed of either POPC/POPS:Chol 3:1, POPC/POPS:POPA 3:2, or POPC/POPS/Chol 3:1:1 led to effects on the ester carbonyl stretching vibrations that differ substantially from those observed upon incorporation of the nAChR into either POPC or POPC/POPA 3:2 membranes. For each POPC/POPS membrane, the lipid ester carbonyl stretching band in the presence of the nAChR exhibits four as opposed to two components that are clearly visible in both the deconvolved (Fig. 5, right panel, spectrum A) and nondeconvolved (Fig. 1.) spectra at 1741, 1729, 1720, and 1711 cm⁻¹. Although not common in FTIR spectra recorded from most lipid membranes, four ester carbonyl stretching vibrations have been detected in spectra of PS bilayers in the presence of calcium (19, 21). In the latter, these vibrations reflect the formation of a complex between Ca²⁺ and the serine carboxylate of PS, leading to a reorientation of the glycerol backbone and consequent change in position of the ester carbonyls relative to the polar-apolar lipid interface. Incorporation of the nAChR into POPS-containing membranes leads to a dramatic increase in the relative intensity of these four components, suggesting that the presence of the nAChR may lead to the formation of a relatively large proportion of Ca²⁺-complexed POPS (compare Fig. 5, spectra B–D in left versus right panels).

Role of Divalent Cations in the Interactions between the nAChR and POPS-containing Bilayers—The altered head group conformation of POPS observed upon incorporation of the nAChR into the POPC/POPS membranes could reflect either an elevated local concentration of Ca²⁺ in the reconstituted versus pure lipid membranes or a direct interaction between the nAChR and POPS that mimics the interactions that have been observed by others between Ca²⁺ and PS in pure lipid systems. To distinguish between these two possibilities, we first examined whether or not Ca²⁺ can induce a change in

### Table I

| Membrane lipid composition | Lipid:protein | \( T_m \) of pure lipid | \( T_m \) of lipid + nAChR | Change in \( T_m \) with nAChR |
|---------------------------|--------------|------------------------|--------------------------|----------------------------|
| POPC/POPA 3:2             | 150:1        | 11                     | 24                       | +13                       |
| POPC/POPS/Chol 3:1:1      | 240:1        | –2                     | Not done                | Not done                  |
| POPC/POPS 3:2             | 153:1        | +1                     | –1                      | –2                        |
| POPC/POPS 3:1             | 152:1        | –3                     | –2                      | +1                        |
| POPC/POPA 3:1             | 147:1        | –6                     | –5                      | +1                        |

**Fig. 5.** The carbonyl stretching region in deconvolved spectra of lipid membranes composed of 3:2 POPC/POPA(A), 3:1 POPC/POPS/Chol(B), 3:2 POPC/POPS(C), 3:1 POPC/POPS(D), and POPC(E). The left panel shows the deconvolved carbonyl stretching band in spectra of pure lipid membranes. The right panel shows the deconvolved carbonyl stretching region in spectra of the same lipids, but in membranes with the nAChR at the lipid:protein ratios specified in Table I. All of the 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 of the other spectra were recorded in the liquid crystal state. All of the spectra have been deconvolved between 1900 and 1300 cm⁻¹ with a factor of 7 and a smoothing parameter of 70%.
FIG. 6. The carbonyl stretching region in deconvolved spectra of 3:2 POPC/POPS membranes hydrated with different H2O buffers (all pH 7). A, 5 mM Tris; B, 5 mM Tris, 250 mM NaCl, 5 mM KCl, 2 mM MgCl2, and 3 mM CaCl2 (i.e. Torpedo Ringer buffer); C, 5 mM Tris, 10 mM EDTA (dashed spectrum) incubated in 5 mM Tris, 20 mM EDTA; D, 5 mM Tris 30 mM CaCl2; E, 5 mM Tris, 250 mM NaCl. The left panel shows the deconvolved carbonyl stretching band in spectra of lipid membranes without incorporated nAChR. The right panel shows the deconvolved carbonyl stretching region in spectra of the same buffers but in the presence of the nAChR. All of the spectra were recorded at 22.5 °C. All of the spectra have been deconvolved between 1900 and 1300 cm⁻¹ with a γ factor of 7 and a smoothing parameter of 70%.

the head group conformation of POPS in a mixed membrane containing both POPS and POPC. In the absence of added divalent cations, spectra of POPC/POPS 3:2 lipid membranes exhibit only two ester carbonyl stretching vibrations (Fig. 6, left panel, spectrum A). In Torpedo Ringer buffer, which contains 3 mM CaCl2 and 2 mM MgCl2, two main C=O stretching vibrations are observed, but there is subtle intensity in the deconvolved spectra, suggesting that a very small proportion of the POPC molecules are complexing with Ca2⁺ (Fig. 6, left panel, spectrum B). In contrast, four clear ester carbonyl vibrations are observed in the spectra of POPC/POPS 3:2 membranes recorded in the presence of 30 mM CaCl2 (Fig. 6, left panel, spectrum D), demonstrating that divalent cations form a complex with POPS leading to an alternative head group conformation of POPS in the POPC/POPS 3:2 membranes, even in the absence of the nAChR.

We examined further the involvement of Ca2⁺ by recording FTIR spectra of the reconstituted POPC/POPS 3:2 membranes in buffer lacking divalent cations, as well as in the presence of EDTA. In the absence of added divalent cations, the four intense ester carbonyl vibrations are still evident when the nAChR is present. The addition of 10 mM EDTA led to a reduction in intensity of the four ester carbonyl bands (Fig. 6, right panel, spectrum C). Interestingly, incubation of the sample in 20 mM EDTA before preparation for FTIR (see “Experimental Procedures”) completely eliminated the four bands, showing that the altered head group conformation of POPS in the reconstituted nAChR membranes requires the presence of Ca2⁺ and is therefore not likely due to a direct nAChR-POPS interaction (Fig. 6, right panel, dashed spectrum).

Note that the relative amount of Ca2⁺-complexed versus noncomplexed PS molecules in the reconstituted membranes is dependent upon the Ca2⁺ to POPS molar ratio (21). We were initially concerned that the observed increase in Ca2⁺-complexed POPS in the reconstituted membranes might reflect a greater Ca2⁺ to POPS ratio in the reconstituted samples because of differences in both sample preparation and lipid concentration. To control for this possibility, we prepared FTIR samples using the same protocol for both the reconstituted nAChR and pure lipid membranes using equivalent molar amounts of lipid in each (see “Experimental Procedures”). The resulting FTIR spectra are identical to those presented in Fig. 6, showing that the increase in the amount of Ca2⁺-complexed POPS in the reconstituted membranes does not reflect an artifact caused by variations in sample preparation.

**DISCUSSION**

The main objective of this work was to determine whether or not negative charge is the defining structural feature of PA that makes it such an effective modulator of nAChR function. Reconstituted membranes composed of PC and the anionic lipid PA are particularly effective at stabilizing the nAChR in a conformation that is capable of undergoing agonist-induced conformational change. The nAChR in PC/PA membranes has slower hydrogen exchange kinetics than the nAChR in PC membranes, likely as a result of a slowing of nAChR internal dynamics (10). Incorporation of the nAChR into membranes composed of POPC and POPA also leads to a substantial increase in both the gel-to-liquid crystal phase transition of the lipid bilayer and the lateral packing density of the lipids (11). In contrast, reconstituted PC or PC/Chol membranes are not as effective at stabilizing the nAChR in a functional conformation (3), nor does the nAChR have a substantial effect on the physical properties of the lipids upon incorporation into these bilayers (11).

Surprisingly, bilayers composed of POPC with either 25 or 40 mol % POPS are not any more effective at stabilizing the nAChR in a resting state than bilayers composed of POPC alone. FTIR difference spectroscopy shows that neither lipid combination can stabilize the nAChR in a conformation that undergoes agonist induced conformational change. Difference spectra for the nAChR in both POPC/POPS 3:1 and POPC/POPS 3:2 are indicative of a completely desensitized or desensitized-like nAChR. Furthermore, reconstitution of the nAChR into membranes composed of POPC/POPS had little effect on the gel-to-liquid crystal phase transition of the bilayer and thus on the physical properties of the membrane. In contrast to PA, there is no bi-directional coupling between the structure/function of the nAChR and the structure of its surrounding lipid environment in membranes composed of POPC/POPS.

The inability of PS and the nAChR to interact with each other in a manner that mimics the interactions that occur between the nAChR and PA clearly shows that a net negative charge is not the sole defining structural feature that makes PA an effective modulator of nAChR function. Although studies with a wider range of anionic lipids is required for a complete understanding of the role of lipid charge in modulating nAChR function, our data highlight the possibility that PA is unique in its interactions with the receptor. Defining the nature of these interactions is important for understanding the mechanisms of lipid-protein interactions at the nAChR. These interactions could also have broader biological significance. Although PA is found at low levels in Torpedo membranes, it is possible that...
there are high local concentrations of PA within the bilayer that influence nAChR function in a post-synaptic membrane (12). The nAChR in neuronal membranes is targeted to lipid rafts (22–24). It is possible that PA acts within rafts or microdomains to modulate nAChR function in vivo. The same mechanisms by which the nAChR influences the physical properties of bilayers containing PA could also allow the nAChR to participate in lipid raft or microdomain formation.

Despite its inability to stabilize a functional nAChR, our data show that POPS interacts with the nAChR in a unique fashion. Reconstitution of the nAChR into membranes composed of POPC/POPS leads to the appearance of four lipid ester carbonyl stretching vibrations near 1741, 1729, 1720, and 1711 cm–1 (Fig. 5, right panel, spectra B–D). Hubner et al. (21) have reported that four lipid ester carbonyl stretching vibrations are observed in spectra recorded from PS membranes in the presence of calcium. These four bands reflect the formation of a complex between Ca2+ and the serine carboxylate of PS, leading to a reorientation of the glycerol backbone and a consequent change in the position of the ester carbonyls relative to the polar-apolar lipid interface. The different positions of the ester carbonyls in the bilayer give rise to variations in their extent of hydration, which in turn gives them distinct absorption frequencies in the IR spectrum. In PS-Ca2+ membranes, the 1741- and 1720-cm–1 peaks originate from the non-hydrogen-bonded and hydrogen-bonded sn-1 ester carbonyl respectively, whereas the 1729- and 1711-cm–1 peaks originate from the non-hydrogen-bonded and hydrogen-bonded sn-2 ester carbonyl, respectively (21). The intensities of the four components reflect the relative proportion of Ca2+-complexed PS.

The altered head group conformation of POPS in the presence of the nAChR is also dependent upon the presence of divalent cations because the spectral features indicative of the formation of this altered conformation are eliminated in the presence of 20 mM EDTA. The finding that divalent cations are still present in the reconstituted POPC/POPS membranes is surprising. In fact, the altered head group conformation of POPS in the reconstituted POPC/POPS membranes is observed even when divalent cations are not added to the FTIR samples. During affinity purification and reconstitution of the nAChR into the POPC/POPS membranes, neither the lipids nor the nAChR are exposed to buffer containing added Ca2++. In fact, all of the solutions used in the reconstitution protocol contain 0.1 mM concentrations of EDTA. The fact that the reconstituted samples exhibit an altered POPS conformation dependent on divalent cations, despite stringent efforts to remove divalent cations (including dialysis five times against 2 liters of divalent cation free buffer with EDTA), shows that either POPS or the nAChR or a combination of both components have an extremely high affinity for Ca2+. Given that the nAChR is a cation-selective ion channel and has a pL of ~5, it is possible the nAChR recruits Ca2+ to the bilayer surface in native membranes leading to an elevated local concentration. Ca2+ may bridge a direct interaction between negatively charged lipids and the nAChR that is formed in native membranes and maintained throughout the affinity purification protocol. The nAChR and/or POPS must have an extremely high affinity for divalent cations.

A second relevant feature of PS lipid bilayers reported by Hubner et al. (21) is that Ca2+ complexation leads to a dramatic increase in the gel-liquid crystal transition temperature. In samples with only a fraction of the PS complexed to Ca2+, two transitions are observed: one transition at the temperature expected for the noncomplexed form of PS and the second at a higher temperature due to the Ca2+-complexed PS (21). In our reconstituted POPC/POPS samples containing the nAChR, only one transition is observed, but the C–H stretching frequency of the lipids in the liquid crystalline phase is always reduced to the frequency of the vibration observed for membranes lacking the nAChR (see in particular Fig. 4C). The degree to which the frequency is depressed may reflect the percentage of complexed PS in the membrane (compare Fig. 4, C and D). The temperature of the gel-to-liquid crystal transition of the structurally altered POPS in our reconstituted nAChR membranes may be higher than the temperature range of our experiment. Heating the samples beyond 35 °C might induce a second transition, bringing the frequency of the C–H stretching vibration closer to that seen for the liquid crystalline state in membranes without the nAChR.

Our results show that the nAChR can interact with two anionic lipids, PA and PS, in specific and distinct fashions. The interactions between the nAChR and PA lead to a functional receptor, whereas the interactions between the nAChR and PS do not. Interactions between the nAChR and PS could, however, still play an important role maintaining membrane structure. The data show that lipid-protein interactions at the nAChR are clearly complex.

Finally, it is interesting to note that mixtures of POPC/POPS/Chol 3:1:1 are effective at modulating nAChR structure and function. The residual amid II intensity observed after exposure of the nAChR in POPC/POPA/Chol 3:1:1 to 2H2O suggests that this membrane exhibits peptide hydrogen exchange kinetics that are intermediate between the kinetics observed for the nAChR in POPC/POPA 3:2 and POPC/POPS samples (Fig. 3, right panel, spectrum B). The nAChR in POPC/POPS/Chol 3:1:1 also exhibits an ability to stabilize the resting conformation of the nAChR that is intermediate between membranes composed of POPC/POPA and those composed of POPC/POPS. The relative proportion of functional receptors is greater than what would be expected for a PC membrane with only 20% Chol (3) or 20% POPS, suggesting that PS and Chol work synergistically to provide a somewhat functional nAChR membrane.

It appears that POPS and Chol act together to create an environment conducive to resting state stabilization. We and others have suggested that membrane fluidity may play a role in modulating the functional state of the nAChR (3, 4, 11). It is possible that the combination of a small head group and negative charge on PA allows mixtures of PA and PC to create an ordered, net negatively charged environment that is similar to the environment found in PC/PA/Chol or POPC/POPS/Chol membranes. Further studies should elucidate how interactions between lipids such as PS and PA as well as bilayer physical properties and divalent cations all work together to create an environment conducive for optimal nAChR function.

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