Correlation of phospholipid structure with functional effects on the nicotinic acetylcholine receptor

A modulatory role for phosphatidic acid

Anil Bhushan and Mark G. McNamee
Department of Biochemistry and Biophysics, University of California, Davis, California 95616 USA

ABSTRACT Fourier transform infrared spectroscopy is used to characterize specific interactions between negatively charged lipids, such as phosphatidic acid, and the purified nicotinic acetylcholine receptor from *Torpedo californica*. The specific interaction of phosphatidic acid with acetylcholine receptor is demonstrated by the receptor-induced perturbation of the lipid ionization state, which is monitored using Fourier transform infrared bands arising from the phosphate head group. The acetylcholine receptor shifts the pK_a of phosphatidic acid molecules adjacent to the receptor to a lower value by almost 2 pH units from 8.5 to 6.6. Decreased pH also leads to changes in ion channel function and to changes in the secondary structure of the acetylcholine receptor in membranes containing ionizable phospholipids. Phospholipase D restores functional activity of acetylcholine receptor reconstituted in an unfavorable environment containing phosphatidylcholine by generating phosphatidic acid. Lipids such as phosphatidic acid may serve as allosteric effectors for membrane protein function and the lipid–protein interface could be a site for activity-dependent changes that lead to modulation of synaptic efficacy.

INTRODUCTION

The biological effects of extracellular signals such as neurotransmitters, hormones, and growth factors are mediated by cell-surface receptor molecules that span the plasma membrane. Endogenous compounds that bind to sites distinct from ligand sites are likely candidates for regulation of activity. Among these endogenous compounds are lipids and their metabolites, which could affect coupling relationships through interactions at the lipid–protein interface. Lipids are essential in providing an appropriate medium for membrane protein folding and assembly and also serve to stabilize proteins against denaturation (1, 2). It also has become evident that a heterogeneous lipid bilayer composition is required to retain full activity of many membrane proteins in reconstituted liposomes (3). For example, negatively charged phospholipids (such as phosphatidic acid) and sterols were shown to be essential for the ion channel activity of reconstituted acetylcholine receptor (3). Binding studies of various lipid species to proteins have established that lipids display different binding affinities. In most cases, the requirement of certain lipids for activation correlates with a higher binding affinity for those specific lipid species at the protein surface (4–6). The greatest specificity for membrane proteins was observed for negatively charged lipids, including phosphatidic acid (7).

In addition to a physiochemical role in modulating membrane structure and protein function as outlined above, specific lipids may play a more direct role in signal transduction mechanisms. In many diverse cell types, extracellular signals cause profound changes in cellular metabolism by generating lipid metabolites through the action of phospholipases. There is increased evidence that phospholipase D–mediated breakdown of cellular phosphatidylcholine yields phosphatidic acid (8, 9). Phosphatidic acid is also synthesized by the action of diacylglycerol kinase on diacylglycerol produced from the cleavage of phosphatidylinositol by phospholipase C. The functional role of endogenously generated phosphatidic acid is largely unknown, but there are indications that phosphatidic acid can interact with phospholipase C to regulate phosphatidylinositol hydrolysis (10) and thus indirectly mediate diverse responses usually associated with phosphatidylinositol modulation (11).

Although correlation of specific lipid modulatory effects with lipid–protein interactions can be made, none of the experiments to date provide a direct link between the biochemical functions of membrane proteins and the structural and dynamic properties of specific membrane lipids. In the experiments described here, we take advantage of a fully defined model system and a biophysical technique, Fourier transform infrared (FTIR)[1] spectroscopy, that has the ability to probe the dynamic molecular structure of lipids. The model system is the nicotinic acetylcholine receptor (AcChoR) from *Torpedo californica,* a ligand-gated ion channel whose function in cholinergic transmission is to transduce the binding of acetylcholine into a large increase in postsynaptic membrane cation permeability (12, 13). The ability to reconstitute AcChoR into a defined lipid environment, where it exhibits all the functional properties of the native receptor, makes it ideally suited for correlation of lipid structure and membrane protein function (1, 14).

Address correspondence to Dr. Mark G. McNamee, Department of Biochemistry and Biophysics, University of California, Davis, CA 95616.

A. Bhushan's present address is Molecular Neurobiology Laboratory, The Salk Institute, 10010 N. Torrey Pines Rd., La Jolla, CA 92037.

[1] *Abbreviations used in this article:* AcChoR, acetylcholine receptor; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; EDTA, tetraacetate; FTIR, Fourier transform infrared; MOPS, *N*-(1-morpholino)-propane-sulfonic acid; SDS, sodium dodecyl sulfate.

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MATERIALS AND METHODS

Acetylcholine receptor purification and reconstitution

A crude membrane preparation enriched in AcChoR was prepared as described by McNamee et al. (14). *T. californica* electroplax tissue was obtained from Winkler Enterprises (San Pedro, CA). AcChoR was extracted and purified from AcChoR-rich membranes by affinity chromatography as described by Bhushan and McNamee (15). The column was extensively washed with phosphatidic acid to ensure complete exchange of native lipids. The AcChoR purity was monitored by sodium dodecyl sulfate (SDS) gel electrophoresis and α-bungarotoxin binding (1). Purified AcChoR was dialyzed for 48 h to remove both cholate and carbamylcholine, and the samples were stored in liquid nitrogen. All synthetic lipids were obtained from Avanti Polar Lipids (Birmingham, AL).

Preparation of membrane samples and FTIR spectroscopy

The purification protocol gave dialyzed membranes with lipid to protein mole ratios between 100 and 200 (15). Reconstituted membranes were dialyzed at various pH values before being centrifuged at 50,000 rpm for 60 min in a rotor (300,000 *g*; model SW 60; Beckman Instruments, Fullerton, CA). The buffer consisted of 100 mM NaCl, 0.1 mM ethylenediaminetetraacetate (EDTA), 0.02% NaN3, 10 mM sodium acetate, 10 mM *N*-(1-morpholino)-propane-sulfonic acid (MOPS), and 10 mM (3-(Cyclohexylamino)-1-propane-sulfonic acid. The aqueous pellet was transferred to a Perkin-Elmer (Norwalk, CT) sealed demountable cell with BaF_2 windows and a 15-μm spacer. In the case where the amide I band was monitored, the pellet was resuspended and incubated in D_2O overnight before centrifuging again. Samples containing only lipids were prepared by drying a chloroform solution of the lipids in a glass test tube with a stream of dry argon and resuspending them in the above buffer.

FTIR measurements were performed with a Perkin-Elmer 1750 FTIR spectrometer interfaced to a 7500 laboratory computer with CDS-3 application software. Fifty scans were collected and averaged for each sample from 4,000 to 900 cm^{-1} using a 2 cm^{-1} nominal resolution in the ratio mode. The spectrometer was continuously purged with nitrogen to remove atmospheric water vapor. No smoothing was applied to membrane spectra; however, the spectrum of buffer was smoothed by using a 25-point Savitsky-Golay algorithm before the subtraction process. Water absorption in all spectra was removed by subtracting incremental amounts of buffer spectrum in the region of interest and the end point determined by obtaining a flat baseline (16).

Areas of the band at 980 cm^{-1} were calculated using a Perkin-Elmer program with a linear baseline connecting the minima at both sides of the band. Due to the variations in the thickness of the sample film, the C-H bending band at 1,460 cm^{-1} was selected as an internal reference because of its pH insensitivity and close proximity to the region of interest. The C-H stretching band at 2,960 cm^{-1} also gave identical results when used as an internal reference. Deconvolution and second derivative analyses were also carried out using the Perkin-Elmer software.

Reconstitution of AcChoR in vesicles for ion flux activity measurements

A mixture of dioleoylphosphatidylcholine (DOPC), cholesterol, and different negatively charged phospholipids in the mole percentage ratio 60:20:20 was used to reconstitute purified AcChoR. Lipid mixtures were prepared in chloroform, and the solvent was removed by drying the lipid in a gentle stream of dry argon. Residual solvent was removed under vacuum for 1 h. Typically, ~60 mg of lipid was used for each reconstitution experiment. The lipid mixture was then dispersed by adding buffer A (100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, 0.02% NaN3, pH 7.4) to give a 60-mg/ml solution and mildly sonicated in a water bath. Solid sodium cholate (Ultrol grade; Calbiochem, La Jolla, CA) was added to the lipid to a final concentration of 6% (wt/vol) cholate. Two parts purified AcChoR (1.5 mg/ml) was combined with one part lipid solution (final cholate concentration 2%), equilibrated at 4°C for 30 min, and dialyzed in buffer A for 48 h with three changes of buffer. The samples were then dialyzed in buffer A at various pH values for 3 h before assaying for activity.

Phospholipase D treatment

A mixture of DOPC and cholesterol in an 80:20 mole ratio was prepared as described above. Two parts of purified AcChoR (1.5 mg/ml) was combined with one part lipid mixture (60 mg/ml) in 6% cholate and 100 units of phospholipase D from *Streptomyces chromofuscus* (type VI) or from cabbage (type I) (Sigma Chemical Co., St. Louis, MO) was added and incubated for 30 min at room temperature. Under these conditions, ~10% of the DOPC was converted to dioleoylphosphatidic acid (DOPA) as monitored qualitatively by thin-layer chromatography. The samples were dialyzed in buffer A for 48 h with three changes of buffer.

Ion flux assay

Carbamylcholine-dependent influx of $^{86}Rb^+$ (New England Nuclear, Boston, MA) into AcChoR-containing vesicles was measured by a manual flux assay (17). In a typical assay, 50 μl of vesicle sample was mixed with 15 μl of a 0.5–1 $\mu Ci/\mu l$ $^{86}Rb^+$ solution with or without 4.33 mM carbamylcholine. Influx was allowed to proceed at a specific temperature (usually 4°C) for 30 s, and then a 50-μl aliquot was applied to a 2-ml disposable Dowex 50W X8 column (BioRad Laboratories, Inc., Richmond, CA) and eluted with 3 ml of salt-free 175 mM sucrose solution. The eluate containing trapped $^{86}Rb^+$ in vesicles was collected in scintillation vials and counted in a Packard liquid scintillation counter without added scintillant.

Other procedures

Thin-layer chromatography was performed in $CHCl_3$-MeOH-CH_3COOH-H_2O (65:15:10:4) using silica gel plates (Baker-Flex Silica Gel IB-F, Baker Chemical Co., Phillipsburg, NJ).

RESULTS

Characterization of membrane samples

Analysis of the protein composition of the reconstituted membrane samples by SDS-polyacrylamide gel electrophoresis revealed only the presence of bands corresponding to the four types of acetylcholine receptor subunits (α, β, γ, δ). To determine qualitatively the homogeneity of the lipid species after purification and lipid exchange, lipids were extracted from membrane samples (18) and analyzed by silica gel thin-layer chromatography. Extracted lipids ran as a single spot corresponding to pure synthetic standards. Extensive characterization of lipid homogeneity by gas–liquid chromatography (2) has shown that complete exchange of native lipids for the desired synthetic lipid occurs when using the same purification protocol. The morphology of the reconstituted membranes was studied previously by electron microscopy of negatively stained samples (19). The mem-

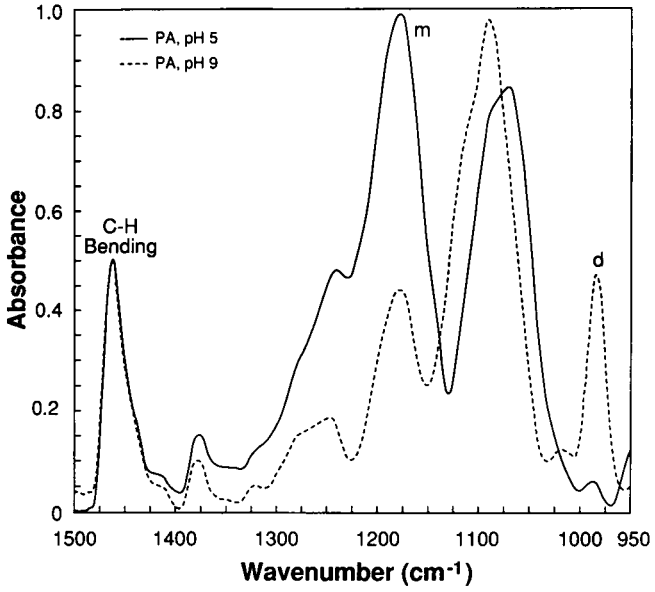

FIGURE 1 FTIR spectra of the 1,500–950 cm^{-1} region of aqueous DOPA at two pH values (5 and 9). Spectra were obtained with 50 scans. Contributions from water have been subtracted and spectra were baseline leveled. Bands assigned to monoanionic and dianionic ionization states have been labeled *m* and *d*, respectively.

branes formed bilayer sheets containing AcChoR with no evidence of membrane vesicles or free receptor.

FTIR analysis

Fourier transform infrared spectra were obtained for lipid-only membranes and for reconstituted membranes containing purified *T. californica* AcChoR at a lipid to protein mole ratio of ~85. DOPA, a negatively charged phospholipid that is known to have a relatively high affinity for the AcChoR (5), was used as the primary lipid to examine specific lipid–protein interactions. The spectral region of 1,500–950 cm^{-1} of the FTIR spectrum contains the characteristic stretching bands of the phosphate ester along with the C-H bending band. Monoanionic and dianionic phosphate monoesters give rise to two bands each in the infrared spectrum of phosphatidic acid (20, 21) and can thus serve as sensitive probes of the ionization state of phosphatidic acid. These bands are easily identified on the basis of their wavenumbers and their pH titration behavior as shown in Fig. 1. The infrared spectra of phosphatidic acid at acidic pH values are dominated by bands arising from monoanionic phosphate group and at high pH values the bands represent the dianionic phosphate species. At acidic pH values, the phosphatidic acid spectra consists of a prominent band at 1,180 cm^{-1} and a broad band centered at 1,065 cm^{-1}. With increasing pH, the 1,180 cm^{-1} band is reduced in intensity, whereas the broad band shifts to 1,085 cm^{-1} and a new band at 980 cm^{-1} appears. The 1,180 and 980 cm^{-1} bands are assigned to the monoanionic antisymmetric stretching mode and dianionic symmetric stretching mode respectively, whereas the broad band at 1,085 cm^{-1} has contributions from the dianionic degenerate stretching mode. This multicomponent broad band that shifts to 1,065 cm^{-1} at acidic pH values is attributed to the symmetric stretch of the monoanionic form.

In reconstituted membranes containing AcChoR, the phosphorylated amino acid side chains on the receptor could possibly contribute to the infrared absorption in the phospholipid headgroup region. However, the seven potential phosphorylation sites on AcChoR (22) are relatively insignificant compared with the number of phospholipids (~85) in the reconstituted sample. The phosphatidic acid concentration also obscures very weak peptide backbone skeletal stretching bands that are better observed in samples reconstituted with other lipids (23). Earlier studies of membrane samples of AcChoR reconstituted in phosphatidylcholine show that the region from 1,250 to 950 cm^{-1} of the infrared spectrum is relatively free from interference bands due to the protein (15). The infrared spectrum of the headgroup region of AcChoR reconstituted in phosphatidic acid is compared to the spectrum for pure phosphatidic acid at the same pH in Fig. 2. There are a number of spectral changes that can be noted upon incorporation of AcChoR. The intensity of the 1,180 cm^{-1} band assigned to the monoanionic antisymmetric stretch decreases in intensity, whereas the 980 cm^{-1} band assigned to the dianionic symmetric stretch is larger. These frequency and intensity changes

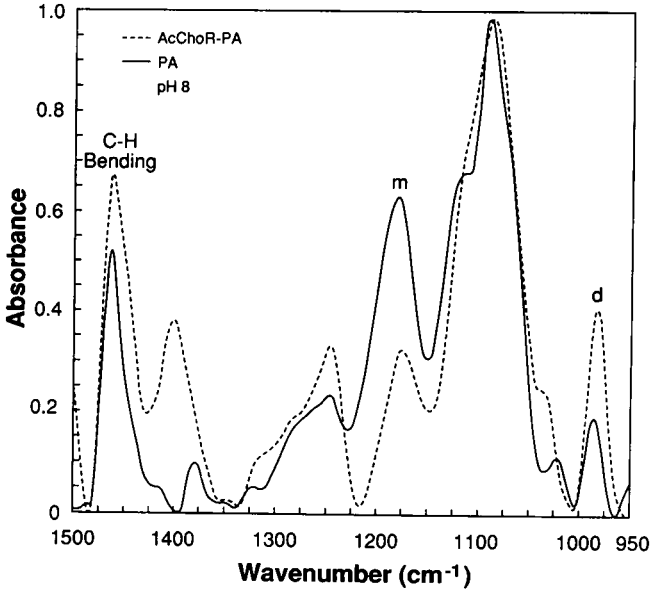

FIGURE 2 FTIR spectra of the 1,500–950 cm^{-1} region of AcChoR reconstituted in DOPA (- - -) and pure DOPA (——) at pH 8. The lipid/protein mole ratio of the reconstituted sample was 85. Spectra were obtained with 50 scans. Contributions from water have been substracted and spectra were baseline leveled. Bands assigned to monoanionic and dianionic ionization states have been labeled *m* and *d*, respectively.

due to incorporation of AcChoR appear to be analogous to pH-induced changes of phosphatidic acid, suggesting that a larger fraction of phosphatidic acid is in the dianionic form in the presence of AcChoR. It thus appears that some of the phosphatidic acid molecules in reconstituted samples behave differently, and perhaps interactions with the receptor molecule could lead to changes in the ionization behavior of these lipids.

pH dependence of FTIR spectra

To study the perturbation of the ionization properties of phosphatidic acid by AcChoR incorporation, the titration behavior of both phosphatidic acid and reconstituted membrane samples was monitored. Samples were incubated at various pH values, and infrared spectra were recorded. The region 1,250–950 cm^{-1} showed large changes in the pH range 5–10. The infrared data were quantitated by using the area of the pH-dependent band at 980 cm^{-1} assigned to the dianionic band since there is no evidence of any underlying components. The C-H bending band at 1,460 cm^{-1} was used as an internal reference, which made comparisons between samples possible over a range of pH values. A similar method of analysis was used by Sanchez-Ruiz and Martinez-Carrion (20) to estimate pK_a values of protein-bound phosphoryl groups. These analyses should be viewed with some caution because of the assumption of linear baselines. However, no large errors are indicated because the pK_a values of pure phosphatidic acid as estimated here are in agreement with previously reported values using other techniques (24).

The ratio of the areas of the dianionic band at 980 cm^{-1} to the C-H bending band at 1,460 cm^{-1} is plotted as a function of pH, and the curves for phosphatidic acid and reconstituted membranes containing AcChoR are shown in Fig. 3. This method permits measurement of the relative concentration of the dianionic species and allows calculation of the pK_a of the samples. The pK_a of pure phosphatidic acid is calculated to be 8.5. It is evident from Fig. 3 that incorporation of AcChoR into phosphatidic acid membranes changes the pH titration behavior significantly, corresponding to an apparent decrease in pK_a. As the lipid/protein ratio of the sample is increased, the titration behavior resembles the pure phosphatidic acid samples (data not shown). It would then appear that the titration behavior of only a fraction of the lipids are perturbed, presumably those that are associated with the protein. Although some lipids show preferential association with proteins, it is assumed that all lipids can exchange between bulk lipid and protein-associated domains (4). The FTIR spectra of AcChoR-containing membrane samples will have contributions from both forms of phosphatidic acid, and the data are analyzed to calculate the titration behavior of phosphatidic acid molecules whose ionization behavior is perturbed due to protein binding.

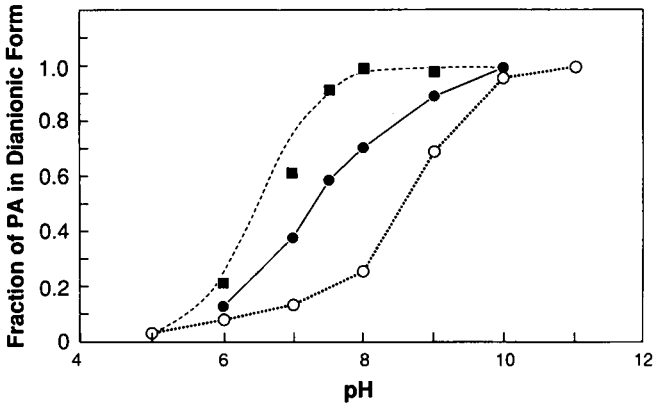

FIGURE 3 Titration behavior of pure phosphatidic acid (○) and reconstituted membrane samples (●) as determined by FTIR. The pH dependence of the ratio of the area of the band assigned to the dianionic phosphate bands (980 cm^{-1}) to the C-H bending band (1,460 cm^{-1}), which is used as an internal standard, is calculated. The calculation of bound phosphatidic acid values (■) was carried out as described in Results. The dashed line represents a simulated curve corresponding to a pK_a of 6.5 for phosphatidic acid molecules bound to the AcChoR.

Calculation of bound phosphatidic acid pK_a

From geometric considerations, ~50 lipid molecules surround the receptor (2, 25). The infrared spectra will represent an average of both bulk lipid and protein-associated domains. The ionization behavior of the lipids in the domain next to the protein (bound) is calculated from the observed spectra as described below.

If α is the fraction of phosphatidic acid molecules that surround the protein, then the integrated intensity of the infrared peak corresponding to the dianionic form of phosphatidic acid (A) would be the weighted ratio of lipids from both domains:

$$A_o = \alpha A_b + (1 - \alpha)A_f, \quad (1)$$

where o, b, and f refer to observed, bound, and free, respectively. The reconstituted membrane samples directly yield the observed fraction of the dianionic form. α can be estimated from previous electron paramagnetic resonance experiments as well as geometric considerations for any given lipid to protein mole ratio (5). The fraction of dianionic form in the free state, A_f, can be expressed as

$$A_f = K_{af}/(K_{af} + [H^+]), \quad (2)$$

using the value of K_{af} obtained for pure phosphatidic acid. Substituting into 1 and rearranging,

$$A_b = (A_o - (1 - \alpha) \times K_{af}/(K_{af} + [H^+]))/\alpha. \quad (3)$$

Calculated values for the bound phophatidic acid molecules are shown in Fig. 3. For the membranes used here, the fraction of lipids surrounding the receptor (α) was estimated to be 0.6 (since the lipid/protein ratio was

determined to be ~85), and the free phosphatidic acid pK_a was calculated to be 8.5. The calculated bound phosphatidic acid values are shown in Fig. 3 superimposed on a simulated curve corresponding to a pK_a of 6.6. Thus, the bound phosphatidic acid molecules have a pK_a that is ~2 pH units lower than bulk phosphatidic acids molecules. The free energy difference between the two pK_a values is 2.8 kcal mol^{-1} and can be attributed to a stabilization of the dianionic form of the phosphatidic acid due to its interaction with the AcChoR.

Salt titration in the range of NaCl concentrations from 0 to 1.0 M at pH 7.4 showed no difference in FTIR spectra for either the AcChoR samples or the pure phosphatidic acid samples (data not shown). Thus, the phosphatidic acid–AcChoR interaction involving the phosphate headgroup is sensitive to pH but cannot be screened by high concentrations of salt.

Effect of other negatively charged lipids

The pH titration data described above provide a convenient method to characterize structural changes in the lipid molecules at the lipid–protein interface. However, correlation of this structural change in the lipid molecules with AcChoR ion translocation function is difficult since the receptor itself contains many pH-sensitive side chains and is known to undergo pH-dependent changes in function (26). The direct effects of pH on the receptor protein need to be differentiated from disruption of specific lipid–protein interactions. This can be achieved by using other negatively charged phospholipids that support the functional activity of the receptor but do not undergo any ionization changes over the pH range used to assay the activity of the receptor and the ionization properties of phosphatidic acid. Changes in the receptor directly due to pH will be manifested as changes in the activity in all samples, whereas changes in specific lipid–protein interactions dependent on the ionization state of the lipid will only be evident in samples containing that specific lipid.

Phosphatidylglycerol and cardiolipin are two negatively charged phospholipids that support AcChoR function (2) and do not undergo any significant changes in ionization in the pH range of 6.5–8 (24). Samples were prepared at high lipid/protein mole ratios containing either phosphatidylglycerol, phosphatidic acid, or cardiolipin in addition to phosphatidylcholine and cholesterol. The complex lipid mixtures are necessary to prepare sealed membrane vesicles suitable for ion flux assays of receptor function as described in Materials and Methods. The carbamylcholine-dependent influx of $^{86}Rb^+$ was assayed at various pH values, and the results are shown in Fig. 4. The samples reconstituted with phosphatidic acid display a sharp increase in ion flux activity as the pH is increased from 6.5 to 8.5. In contrast, the activity of samples reconstituted with phosphatidylglycerol and cardiolipin do not change significantly over the same pH range. Thus, the pH dependence of ion flux appears to be correlated with the ionization state of phosphatidic acid and is independent of the protein ionization state over the limited pH range used here.

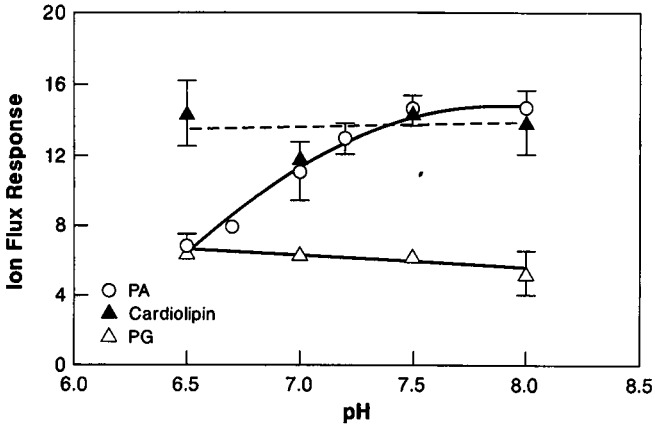

FIGURE 4 pH dependence of ion channel function in different negatively charged lipids. Purified AcChoR-containing membranes were reconstituted into a mixture of DOPA, cholesterol, and a negatively charged phospholipid (60:20:20 mole ratios), consisting of either phosphatidic acid (○), cardiolipin (△), or phosphatidylglycerol (△). The reconstituted membranes were tested for carbamylcholine-induced $^{86}Rb^+$ influx as described in Materials and Methods. Values of the ion flux response are expressed as [cpm(+carb) − cpm(−carb)] × 1,000/cpm (equil), where cpm(+ or −carb) represents ion flux after 30-s incubation with or without carbamylcholine and cpm (equil) represents cpm after 48-h incubation with $^{86}Rb^+$. Results are shown as means ± SD for three sets of samples assayed in triplicate.

Protein secondary structure changes

Aspects of AcChoR protein secondary structure were monitored in reconstituted AcChoR–phosphatidic acid membranes over the pH range of 6.5–8.0 using the amide I band in the FTIR spectra. The amide I band has been shown to be a good measure of the relative secondary structures of proteins (27, 28). The deconvolved and second derivative amide I spectra shown in Fig. 5 have three major bands. The 1,653-cm^{-1} band is assigned to α-helix, the 1,643-cm^{-1} band to disordered, and the 1,631-cm^{-1} band to β-structure (29). Both the deconvolved and second derivative analyses show a relative increase in β-structure, and disordered band intensities relative to the α-helical band as the pH (pD) is raised over the range of the ionization of phosphatidic acid. Both H_2O and D_2O samples showed similar relative changes in secondary structure. Only the relative changes in secondary structure were measured and not the absolute secondary structure content. Negatively charged phospholipids at pH 7.4 previously have been shown to increase the β-structure content of reconstituted AcChoR based on analysis of specific FTIR peaks associated with the protein (23). Based on the pH dependence of the interactions monitored here, ionic interactions between the dianionic phosphate head group and

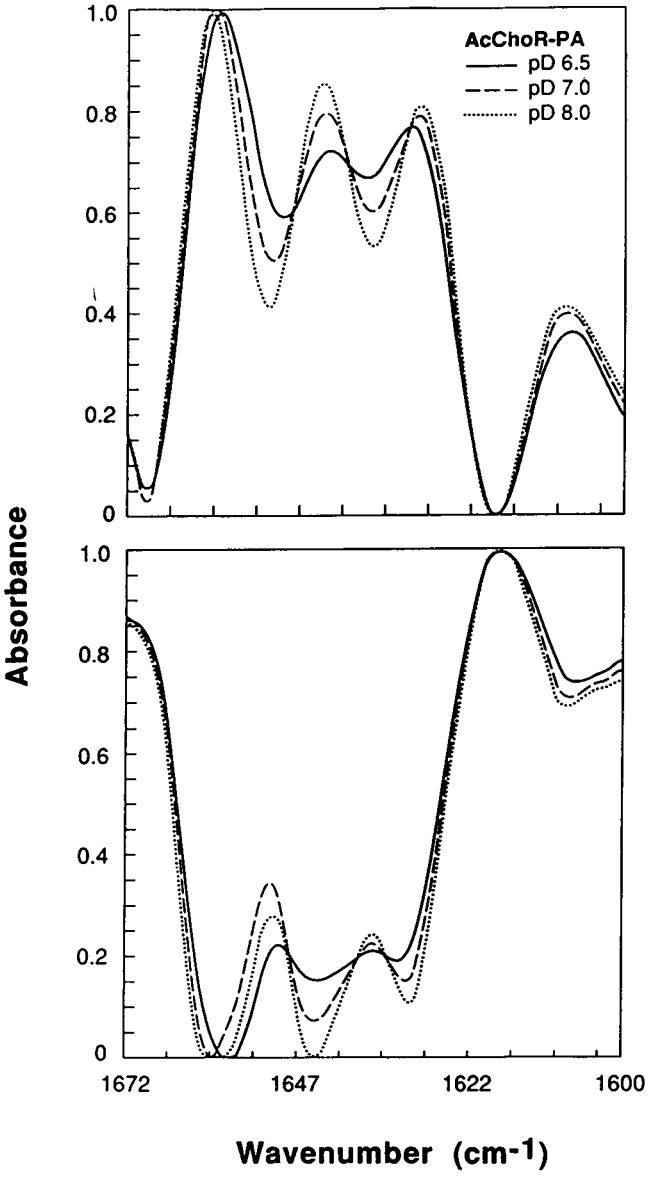

FIGURE 5 Deconvoluted (*top*) and second derivative spectra (*bottom*) of the amide I region of AcChoR reconstituted in phosphatidic acid and incubated in buffer made of D_2O (100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, 0.02% NaN3) at the pD values as indicated in the figure. Deconvolution parameters used were σ = 8.0 cm^{-1} (half-width at half height) and k = 3.0 (relative reduction in bandwidth).

the receptor appear to be stabilized and may be important in maintaining the receptor in an activatable state.

Effect of phospholipase D

Acetylcholine receptor reconstituted in phosphatidylcholine and cholesterol and in the absence of any negatively charged lipids does not exhibit ion translocation function (3). These membrane samples when incubated with phospholipase D from *S. chromofuscus*, which is active at neutral pH, dramatically regained ion translocation function as shown in Fig. 6. Under the conditions of the experiments, ~10% of the phosphatidylcholine was converted into phosphatidic acid as judged qualitatively by thin-layer chromatography analysis. In contrast, phospholipase D from cabbage, which is active only at acidic pH values, had no effect on the ion translocation function of AcChoR.

DISCUSSION

The AcChoR has been shown to be preferentially solvated by negatively charged phospholipids relative to zwitterionic phospholipids using electron paramagnetic resonance and other spectroscopic techniques (4, 5, 7). The higher binding affinity for negatively charged phospholipids, such as phosphatidic acid, appears to have functional consequences since the same lipids are essential for supporting the ion channel activity of the receptor (3, 30). The AcChoR-phosphatidic acid system is an experimentally convenient model system for characterizing specific lipid–protein interactions and for correlating these interactions with the role of these specific lipids in regulating the activity of membrane proteins.

Previous investigations of lipid–protein interactions in membranes using FTIR have exploited the phase transition properties of lipid molecules to determine the effect of protein incorporation on the behavior and organization of lipids (29). Although the thermotrophic behavior of lipids is a useful indicator of the general organization of lipids, the disadvantage of this approach is the inability to relate the melting curves that are generated

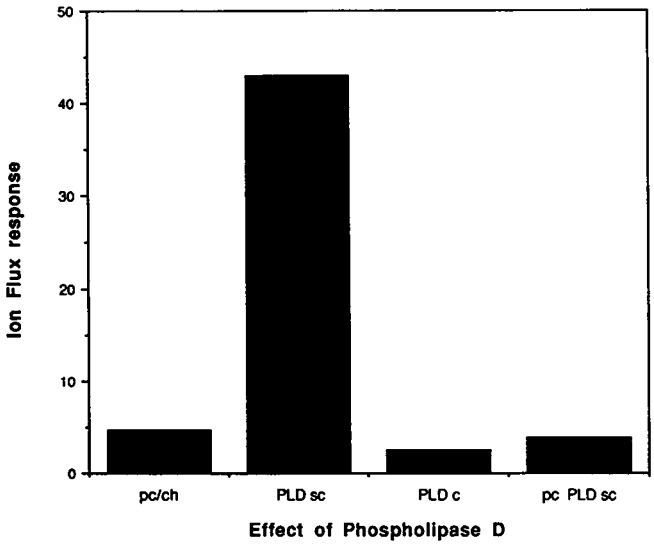

FIGURE 6 Effect of phospholipase D on AcChoR. AcChoR was reconstituted with phosphatidylcholine and cholestrol at an 80:20 mol% ratio (pc/ch) or in pure phosphatidylcholine (pc). Some samples were reconstituted in the presence of various forms of phospholipase D from *S. chromofuscus* (PLD sc) or cabbage (PLD c) and ion flux assays conducted and ion flux responses normalized to the size of the vesicles as described in Results. The ion flux response is expressed as described in Fig. 4. One sample prepared without cholesterol (pc PLD sc) shows the dependence of the flux response on cholesterol in addition to the presence of negatively charged lipids.

(due to the lack of models) to structural information that will identify the sites of specific lipid–protein interactions. In contrast, the three distinct structural domains of the phospholipid molecule (the acyl chains, the interfacial region, and the headgroup region) give rise to characteristic infrared bands (31), which can be used to study the chemical structure and properties of potential interaction sites. Interactions between AcChoR and zwitterionic lipids, such as phosphatidylcholine, were analyzed by examining the regions in the infrared spectra that are typical of the distinct structural regions of the lipid molecule (15).

The experiments reported here clearly demonstrate that FTIR spectroscopy of membrane lipids can be used to characterize molecular aspects of specific lipid–protein interactions. The shift in phosphatidic acid–apparent pK_a induced by the AcChoR can be interpreted most readily as an effect of the protein on the phosphatidic acid molecules at the lipid–protein interface. Although a more generalized effect of the protein on the overall two-dimensional lipid charge distribution in the membrane cannot be ruled out, electron paramagnetic resonance data suggest that the existence of distinct bulk lipid and protein-associated lipid domains is reasonable. The pH-dependent changes in the ion channel activity of reconstituted membranes containing phosphatidic acid correlate directly with the calculated ionization properties of the protein-associated phosphatidic acid molecules. In contrast, reconstituted membranes containing acidic phospholipids that do not undergo a change in ionization over the same pH range show no pH-dependent changes in activity.

These results suggest that the ionization properties of negatively charged phospholipids play a key role in modulating the activity of membrane proteins. The binding specificity of the AcChoR for negatively charged phospholipids could be due to the interaction with positively charged amino acids that are clustered at the end of the postulated transmembrane segments (32). A moderately conserved cluster of positive changes immediately following the third postulated transmembrane crossing on the cytoplasmic side has been suggested as a potential interaction site for negatively charged lipids (Butler, D., and M. McNamee, manuscript submitted for publication). Chemical modification studies are underway to determine the role of these amino acids in perturbing the ionization state of negatively charged phospholipids. Another important element in determining the specificity could be the favorable interaction of the dipole that results from the aligned peptide units of the α-helix and the charged phosphate lipid headgroups resulting in perturbed pK_a's and stabilization of the helices (33, 34).

The secondary structure changes associated with binding of phosphatidic acid to AcChoR involve increases in the β-structure content. These data are consistent with an earlier study of the relationship between AcChoR secondary structure and lipid composition in a reconstituted system (23). A model has implicated salt bridges between phosphorylated amino acids and positively charged Arg and Lys residues as a mechanism by which the β-structure can be stabilized (35). Interestingly, the effect of protein phosphorylation on secondary structure of myelin basic protein was to induce β-structure (36). It is tempting to speculate that binding of phosphatidic acid may be analogous to phosphorylation in the sense that the addition of highly charged phosphate molecules alters the structure of the protein and thereby regulates its function.

The ion flux responses of samples reconstituted with cardiolipin are consistently higher than those reconstituted with phosphatidylglycerol. The difference may be attributed partially to the two phosphate groups of cardiolipin, possibly allowing cardiolipin to interact more strongly with AcChoR in a manner analogous to the dianionic form of phosphatidic acid. Infrared data suggest that the two phosphate groups of cardiolipin are conformationally nonidentical when incorporated into the bilayer (37). A significant difference between cardiolipin and the other lipids is the number of acyl chains. Cardiolipin with its four acyl chains may be able to interact more effectively with a hydrophobic domain on the protein that also appears to be important in regulating ion channel activity (33, 38). In a recent study of amino acid transport in reconstituted systems, Lin et al. (39) showed that the rate of sodium-dependent amino acid uptake was greatly enhanced by cardiolipin and phosphatidic acid but not by other negatively charged lipids.

The ability of phosphatidic acid to specifically mediate allosteric interactions is important because the amount and concentration of phosphatidic acid can be controlled to a large degree. Agonist binding to receptors coupled to G proteins can induce phosphatidylcholine hydrolysis by activation of phospholipase D. The amounts of phosphatidic acid used here in the reconstituted system are high compared with likely physiological concentrations, but functional membranes have been prepared with much lower concentrations in the range of 5% (Fong, T. M., and M. McNamee, unpublished observations). Phosphatidic acid is rapidly interconverted to other lipid metabolites such as diacylglycerol by specific enzymes, and thus its concentration in the membrane can be regulated (9). The ability of phospholipase D to regulate AcChoR function provides a possible link between G protein–coupled receptors and ion channel receptors.

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