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Differential scanning calorimetry and Fourier transform infrared analysis of lipid–protein interactions involving the nicotinic acetylcholine receptor

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Lipid–protein interactions were studied using *Torpedo californica* acetylcholine receptor (AChR) as a model system by reconstituting purified AChR into dielaidoylphosphatidylcholine (DEPC, 18:1 *trans*-9,10) membranes. The structural and thermodynamic behavior of lipids in the vicinity of the protein were studied by differential scanning calorimetry and Fourier transform infrared spectroscopy. The effects of AChR on the thermodynamic parameters associated with lipid phase transitions were to reduce the enthalpy change, lower the transition temperature and reduce the cooperative behavior of the lipid molecules. A stoichiometry of approx. 95 lipids per AChR molecule was found by simulating the decrease in enthalpy in terms of a simple model in which a fixed number of lipid molecules are prevented from undergoing a cooperative phase transition. In parallel, the vibrational spectra of pure DEPC and AChR reconstituted in DEPC membranes at various lipid to protein ratios were examined. Profiles of the 3000–2800 cm^{-1} C-H stretching region and 1350–950 cm^{-1} characteristic of the headgroup region of the lipid exhibit little sensitivity to protein/lipid ratio reflecting weak interaction of AChR with DEPC. The lipid carbonyl on the other hand appear to be increasingly hydrogen bonded in the presence of AChR. The results provide new information about the size and physical state of the motionally restricted lipid environment that surrounds the acetylcholine receptor. The results are discussed in the context of lipid-mediated alterations in acetylcholine receptor function.

Introduction

It has become increasingly apparent from biochemical and biophysical studies that membrane protein function is dependent on the lipid environment [1–3]. Although the functional effects of lipid physical properties on membrane proteins have been extensively studied, the structural and dynamic properties of lipids at the lipid/protein interfaces are not well characterized. Based on the different sensitivities of various membrane proteins to the lipid composition, many aspects of lipid–protein interactions must be characterized in several systems before a generalized and useful model of lipid–protein interactions can be postulated.

The ability to reconstitute nicotinic acetylcholine receptor (AChR) from *Torpedo californica* in a defined lipid environment, where it exhibits all the functional properties of the native receptor, makes the AChR an ideal model system [4]. The ability of the receptor to undergo both activating ligand-mediated allosteric state transitions and channel gating is highly dependent on the lipid environment [5,6]. Although no absolute lipid requirement has been demonstrated, it appears that sterols and phosphatidic acid (or other negatively charged lipids) are critically important for successful reconstitution of ion channel activity [5,7].

The interaction of AChR with various lipid systems has been studied by spectroscopic techniques such as fluorescence quenching [8], electron paramagnetic resonance (EPR) spin-labeling methods [5,9] and Fourier transform infrared spectroscopy (FTIR) [7]. These studies focused on the selectivity of AChR for various lipid species and demonstrated that phosphatidic acid, sterols and fatty acids have a relatively high binding affinity for AChR compared to phosphatidylcholine. The fluo-

Abbreviations: AChR, acetylcholine receptor; BSA, bovine serum albumin; Carb, carbamylcholine chloride; EDTA, ethylenediaminetetraacetic acid; DEPC, dielaidoylphosphatidylcholine; DSC, differential scanning calorimetry; DTT, dithiothreitol; EPR, electron paramagnetic resonance; Mops, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; FTIR, Fourier-transform infrared.

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rescence quenching data provide evidence that sterols may occupy sites at the protein/lipid interface that are not available to phospholipids. The FTIR data demonstrate that different lipids alter the relative amounts of alpha helix and beta sheet structure of the reconstituted AChR [7].

All these experimental approaches and many others on different membrane proteins (for reviews see Refs. 10 and 11) suggest relatively weak association of zwitterionic lipids (such as phosphatidylcholine) with membrane proteins in contrast to several clear examples of sterol or negatively charged lipid interactions. However, lipids such as phosphatidylcholine are generally used as the baseline or control lipid in the experiments and little attention is given to the dynamics of phosphatidylcholine–protein interactions. In view of the ubiquitous presence of phosphatidylcholine in nearly all types of both native and reconstituted membrane preparations, a more quantitative analysis of the interactions is essential. Characterization of the interactions enables us to estimate the total number of lipid molecules in contact with the intramembranous surfaces of AChR and provides a better framework for studying specific interactions. Evaluation of these interactions at a molecular level could prove to be important for understanding the role of lipid–protein interactions in determining the structure and function of biological membranes.

Differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR) together provide a powerful approach to study the influence of proteins on the organization and dynamics of lipid components in a reconstituted system. While FTIR provides structural and motional information resulting from perturbation of the lipid molecules, DSC provides information on the thermodynamic basis for the interactions. In this paper, we present a detailed study of the lipid phase transition properties of dielaidoylphosphatidylcholine (DEPC) reconstituted with AChR at various lipid to protein mole ratios as studied by DSC. Important thermodynamic parameters that characterize this phase transition are reported. Dielaidoylphosphatidylcholine was chosen as the host lipid since it undergoes a phase transition at a convenient temperature (12°C), has been used in reconstitution experiments before to study secondary structure of AChR [12], and is the simplest lipid that sustains ligand-induced changes in acetylcholine receptor conformation [5].

Materials and Methods

Acetylcholine receptor purification and reconstitution. A crude membrane preparation partially enriched in AChR was prepared as described [4]. *Torpedo* electroplax tissue was obtained from Dr. H. Wang, University of California, Santa Cruz, CA. AChR was purified from AChR-rich membranes by affinity chromatography [5] with a modification of the affinity column preparation to take advantage of significant cost savings and product reliability made possible by using Affi-Gel 10 instead of Affi-Gel 401. Briefly, Affi-Gel 10 (Bio-Rad, Richmond, CA) was suspended in 50 ml of 0.054 M cystamine hydrochloride in 20 mM Mops at pH 7.4 for 1 h in a 3 × 20 cm Bio-Rad Econo-column. The gel was washed with water and resuspended in 50 ml 0.1 M DTT in 0.1 M Mops at pH 8.0 for 30 min. The suspension was then allowed to pack and washed with water to remove DTT. The gel was then suspended in 0.1 M Mops at pH 7.4 and 500 mg of bromoacetylcholine bromide [13] was added with vigorous mixing and incubated for 30 min. The column was washed with 100 ml of water, and residual sulfhydryl residues were alkylated by adding 50 mg of iodoacetamide. The column was washed with 0.1 M sodium acetate at pH 4 for storage at 4°C and washed with buffer A (100 mM NaCl, 10 mM Mops, 0.1 mM EDTA, 0.02% NaN_3 at pH 7.4) before use.

All steps were carried out at 0–4°C. Crude membranes were diluted with buffer A to a protein concentration of 2 mg/ml and solubilized by adding sodium cholate, with gentle stirring, to a final concentration of 1.2% (w/v) cholate. This mixture was centrifuged in a Beckman Type 35 rotor at 35000 rpm for 60 min (95000 × *g*). The supernatant was filtered through four layers of cheesecloth and applied to the affinity column. The column was washed with 3 column bed volumes of a solution containing 2 mg/ml of the desired lipid and 1% cholate in buffer A and allowed to stand for 4 h or overnight to ensure complete exchange of native lipids for the desired synthetic lipid. The column was finally washed with 100 ml of 0.1 mg/ml desired lipid and 0.5% (w/v) cholate in buffer A. (Ultrol grade cholate (Calbiochem, San Diego, CA) was used for washing and subsequent resolubilization of purified receptor during reconstitution.) AChR was then eluted by applying 100 ml of final wash containing 10 mM carbamylcholine (Carb) as eluent. Protein concentration was determined by A_{280} (protein mg/ml = $A_{280} \times 0.6$) and fractions were pooled to achieve a protein concentration of 1 mg/ml and dialyzed for 48 h at 4°C against 4 l of buffer A with three buffer changes. For calorimetric experiments the pooled fractions were concentrated about 4-fold in Centriprep-30 concentrators (Amicon, Danvers, MA) before dialysis. The AChR purity was monitored by SDS gel electrophoresis and α-bungarotoxin binding (see Ref. 14). All synthetic lipids were obtained from Avanti Polar Lipids (Birmingham, AL) and Asolectin from Associated Concentrates (Woodside, NY).

Reconstitution of AChR at different lipid/protein molar ratios. The purification protocol described above gives dialyzed membranes with lipid to protein mole ratios (ϕ) between 100 and 200. Membranes of higher

lipid to protein ratios were prepared by varying the amounts of resolubilized membranes and additional lipids in order to keep the total lipid concentration constant. Typically, the lipid concentration of the membrane sample was determined and a solution of the desired lipid of the same concentration was prepared by drying the lipid to a thin film in the bottom of a small glass vial using a gentle stream of dry argon. Residual solvent was removed by vacuum for 1 h. The lipid was then dispersed by adding buffer A and mildly sonicating in a water bath. Sodium cholate (Ultrol grade) was added to the lipid to a final concentration of 0.5% (w/v) cholate. Dialyzed membranes were also resolubilized with sodium cholate at the same concentration of 0.5% cholate. Varying amounts of both solubilized membranes and lipid solution were combined to achieve the desired lipid to protein mole ratio. The combined mixture was equilibrated at 4°C for 30 min and dialyzed in buffer A for 48 h with four changes of buffer. Phospholipid concentration was determined by total phosphate content [15]. Protein concentration was determined by the Lowry method [16].

Sucrose density gradient centrifugation. After reconstitution, the incorporation of proteins into membranes was monitored by discontinuous flotation gradients. 1 ml of the sample was thoroughly mixed with 1 ml of sucrose (60% w/w) in buffer A in a 12 ml centrifuge tube, 2 ml each of 30%, 20%, 10%, and 2.5% sucrose (w/w) was successively layered, followed by 1 ml of buffer A. The samples were centrifuged at 30000 rpm for 90 min in a Beckman SW 40 rotor. Fractions of 0.5 ml were collected and analyzed for lipid and protein content.

Electron microscopy. Samples were examined by negative stain electron microscopy. 10-μl samples were applied to 400-mesh copper grids which had been coated with parlodian and shadowed with carbon. The samples were blotted, washed, stained with 0.5% uranyl acetate, and examined in a Phillips TEM-400 electron microscope at 100 kV.

Differential scanning calorimetry. Calorimetric experiments were performed with a Hart Scientific (Orem, UT) Differential Scanning Calorimeter. All calorimetric scans were performed at a scanning rate of 10 C°/h. Lipid concentrations were in the range of 2–3 mg/ml. At least two runs were made with each sample. A Fortran program was written to perform all data analysis. The area of the calorimetric peak was determined by numerical integration. The degree of transition at temperature T, $\theta(T)$, was determined by integrating the excess heat capacity curves to T and dividing by the area under the curve [17]:

$$\theta(T) = \frac{\int^{T} \Delta C_p(T)\, dT}{\int^{\infty} \Delta C_p(T)\, dT} \quad (1)$$

The midpoint temperature T_c, of the transition is defined as the temperature at which $\theta = 1/2$. The van't Hoff enthalpy ΔH_{vH}, was obtained from the derivative of the $\theta(T)$ curve at the midpoint of the transition ($\theta = 1/2$):

$$\Delta H_{vH} = 4RT_c^2 (d\theta/dT)T_c \quad (2)$$

Since $\Delta S^0 = ((\Delta C_p/T)\, dT)$, the ΔC_p vs. T curve was converted into a $\Delta C_p/T$ vs. T curve. The area under such a curve provided a measure of the entropy change [18].

FTIR spectroscopy. Reconstituted membranes (1 ml at 1 mg/ml AChR) were diluted to 3.5 ml in buffer A and centrifuged at 50000 rpm for 60 min in a Beckman SW 60 rotor (300000 × g). In experiments where D_2O was used,the samples were resuspended in D_2O, allowed to equilibrate overnight, and then centrifuged again. The aqueous pellet was transferred to a Perkin-Elmer sealed demountable cell with BaF_2 windows and a 15-μm spacer. FTIR measurements were performed with a Perkin-Elmer 1750 FTIR spectrometer, a 7500 laboratory computer, and CDS-3 application software using a 2 cm^{-1} nominal resolution in the ratio mode. Fifty scans were collected and averaged for each sample from 4000 to 800 cm^{-1}. The spectrometer was continuously purged with nitrogen to remove atmospheric water vapor. Water absorption in all spectra was removed by subtracting incremental amounts of buffer A spectrum in the region of interest and the end point determined by obtaining a flat baseline [19]. Spectral deconvolution was performed by Perkin-Elmer's ENHANCE function, which is analogous to the method Kauppinen et al. [20].

Other procedures. Thin-layer chromatography was performed in $CHCl_3$/ MeOH/ CH_3COOH/ H_2O (65 : 15 : 10 : 4, v/v) using a silica gel plate (Baker-flex silica gel IB-F from Baker Chemical Co., Phillipsburg, NJ).

Results

Characterization of reconstituted membranes

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. The functional integrity of the receptor protein was assessed by using a manual ion flux assay to measure carbamylcholine-stimulated influx of $^{86}Rb^+$ [4]. Samples used in the spectroscopic experiments were in the range of lipid to protein ratios that do not form sealed vesicles [21] and could not be used directly for flux studies. Samples were re-reconstituted in asolectin, a soybean lipid mixture that has been shown to provide a proper lipid environment for the receptor to undergo ion channel activation [22,5]. The re-reconstitution

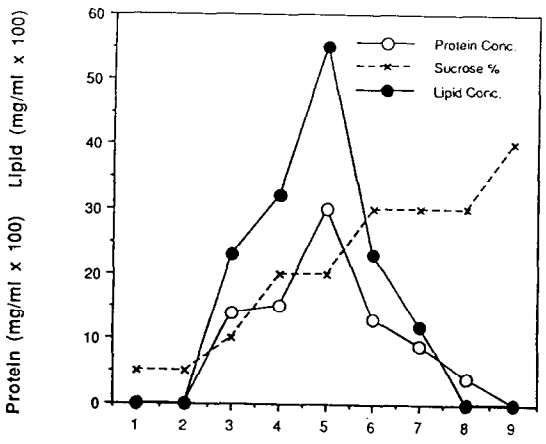

Fig. 1. Sucrose density gradient centrifugation of an AChR-DEPC sample at a lipid to protein mole ratio of 1000 in discontinuous sucrose gradients. AChR-DEPC samples were reconstituted and placed at the bottom of the centrifuge tube for flotation analysis in sucrose gradients as described in Materials and Methods. Samples with lipid to protein ratios below 1000 displayed similar profiles. The gradient was fractionated and both the total lipid (●) and protein content (○) were measured on each sample. Sucrose density (×) was measured by refractometry.

method has been shown to provide a reliable indicator of receptor functional integrity [14]. All samples showed the ability to undergo activating ligand-induced ion translocation.

To qualitatively determine the homogeneity of the lipid species after purification, lipids were extracted from membrane samples [22] and analyzed by silica gel thin-layer chromatography in $CHCl_3$/ MeOH/ CH_3COOH/ H_2O (65 : 15 : 10 : 4, v/v). Extracted lipids ran as a single spot corresponding to the control pure lipid samples. Extensive characterization of lipid homogeneity earlier [14] has shown that complete exchange of native lipids for the desired synthetic lipids occurs by using the protocol described here. Detergent removal was quantitatively monitored by incorporating radiolabelled cholate during reconstitution. Less than 1% of the initial cholate was detected at the end of dialysis.

Incorporation of proteins into membranes was determined by subjecting the reconstituted samples to discontinuous sucrose density gradients. The specific density of the reconstituted membranes was between that of pure phospholipid and what is assumed to be a typical value for proteins, 1.4 g/ml. Fig. 1 shows the

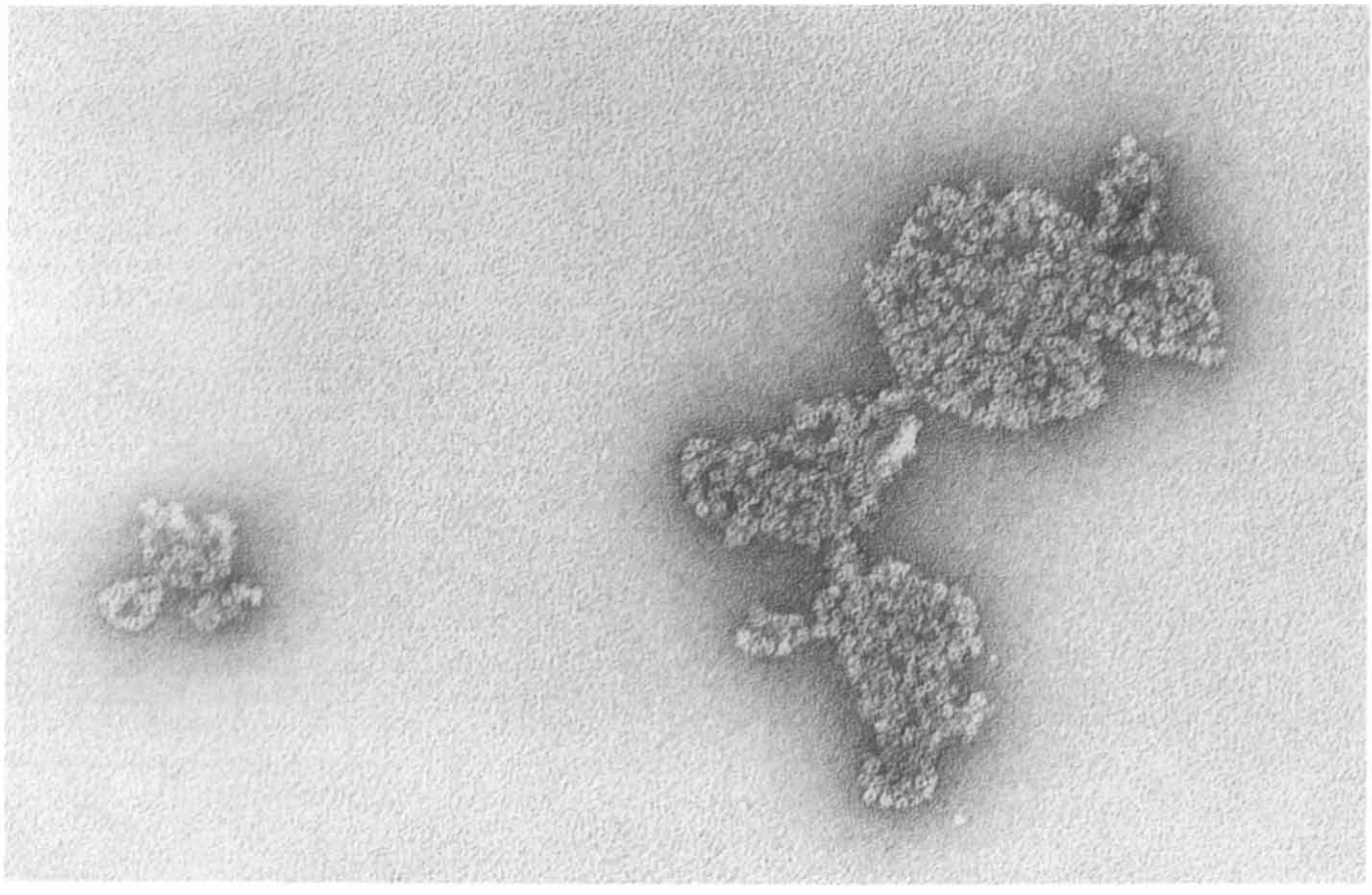

Fig. 2. Electron micrographs of negatively-stained samples of DEPC-AChR reconstituted at a lipid to protein mole ratio of 400 as described in Materials and Methods.

sucrose density pattern of one of the reconstituted samples. It is clear that the reconstituted lipid protein complexes banded into one population containing all the protein and phospholipids. The flotation method eliminated the possibility that free proteins will co-migrate with lipids as the protein moves toward the bottom of the tube. In the absence of protein all phospholipids were found at the 5% and 2.5% (w/w) sucrose interface (data not shown). No effect was seen on the sucrose density gradient profiles of AChR-DEPC samples when dialyzed above (15 °C) and below (4 °C) the main phase transition temperature (12 °C).

The morphology of the reconstituted membranes was studied by electron microscopy of negatively stained samples. The results for AChR-DEPC are shown in Fig. 2. The gross morphology of all samples indicate that the membranes form bilayer sheets with no evidence of membrane vesicles. The acetylcholine receptor molecules are clearly defined as the rosette type structures in the micrographs. There is little contamination by either free receptors or lipid vesicles devoid of receptors, which are often observed in samples at higher lipid to protein ratios [21,14], confirming the evidence deduced from sucrose gradients.

Differential scanning calorimetry of AChR-DEPC samples

DSC measurements of the gel to liquid-crystalline phase transition of AChR-DEPC membranes at different lipid to protein ratios are shown in Fig. 3. The data in Fig. 3 were obtained from heating scans although results were similar for cooling scans at the same scan rate. Repeated scans were identical implying that the sample was stable over the time course of the experiments. These data illustrate several facts about phosphatidylcholine phase behavior in membranes containing AChR. First, the main phase transition was broadened with increasing protein content relative to membranes containing no protein. The broadening of the lipid phase transition indicates that AChR disrupts the cooperative behavior of the lipid molecules. Second, the main phase transition peak height decreased with decreasing lipid to protein ratio. Finally, a decrease in the mid-point of the temperature of the main phase transition became evident at lower lipid to protein ratios. All of these qualitative indicators demonstrate that the incorporation of AChR has a pronounced effect on the thermotropic behavior of the phospholipid molecules.

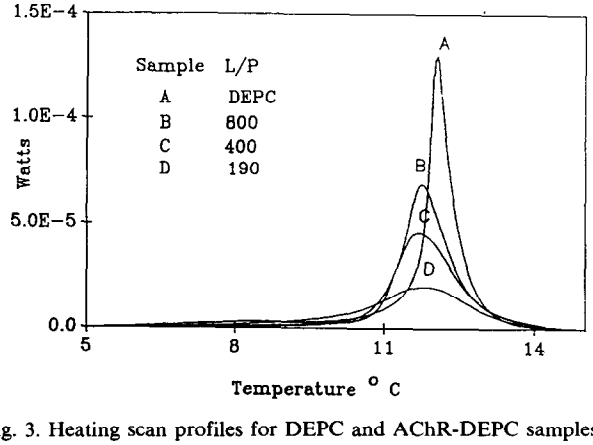

Fig. 3. Heating scan profiles for DEPC and AChR-DEPC samples at varying lipid to protein molar ratios. All the scans were run at a scanning rate of 10 C°/h in buffer A using a Hart differential scanning calorimeter. All sample concentrations in the calorimeter were 1.9 mg DEPC/ml. The protein concentration varied depending on the lipid to protein mole ratio.

TABLE I

Lipid phase transition properties of DEPC in reconstituted acetylcholine receptor-DEPC membranes at different lipid to protein mole ratios

The enthalpy change (ΔH_{cal}), the transition temperature (T_c), the van't Hoff enthalpy (ΔH_{vH}) and the enthalpy change (ΔS) were calculated as described in Materials and Methods for reconstituted membranes prepared at different lipid to protein mole ratios. The cooperative unit is defined as the ratio of $\Delta H_{vH}/\Delta H_{cal}$.

L/P (mol/mol)	ΔH_{cal} (kcal/mol)	T_c (°C)	ΔH_{vH} (kcal/mol)	ΔS (cal/mol)	Cooperative unit
190	3.67	11.79	259.87	12.89	70.81
250	4.62	11.84	286.99	16.20	62.12
400	5.64	11.95	380.14	19.80	67.40
500	6.23	11.96	408.86	21.86	65.63
600	6.62	12.01	466.24	23.23	70.43
800	6.45	11.96	481.99	22.64	74.73
Pure DEPC	7.4	12.22	742.02	25.92	100.41

The degree of transition $\theta(T)$ was obtained by integration of the heat capacity curves and was used to calculate melting temperature (T_c) and van't Hoff enthalpy ΔH_{vH} as described under Materials and Methods. Table I summarizes the thermodynamic data derived from the heat capacity curves of Fig. 3. The phase transition enthalpy change ΔH_{cal} was determined from the area under the heat capacity peaks, plotted as a function of lipid to protein mole ratio and shown in Fig. 4. The decrease in the phase transition enthalpy with decreasing lipid to protein mole ratio indicates that the protein molecules are preventing some phospholipid molecules from undergoing the lipid phase transition. The dependence of ΔH_{cal} on the lipid to protein mole ratio was simulated to a first approximation by a simple equation:

$$\Delta H_{cal} = ((L/P - m)/(L/P))\Delta H_{pure} \quad (3)$$

where m is the number of lipid molecules prevented from participation in the gel to liquid-crystalline transition per protein molecule. As shown in Fig. 4, the fit of Eqn. 3 to the data indicates that each AChR molecule

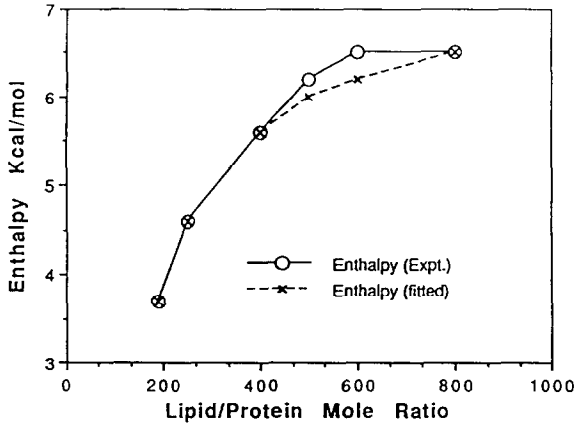

Fig. 4. The experimentally determined enthalpy of the DEPC main phase transition is plotted versus the lipid to protein molar ratio (○———○). The fit of the data to Eqn. 3 (see Results) is illustrated by the dashed line (×- - - - - - -×).

prevents 95 ± 5 DEPC molecules from participating in the phase transition.

Fourier transform infrared spectroscopy

FTIR spectroscopy was used to monitor the molecular conformation of DEPC in AChR-DEPC reconstituted membranes at different lipid to protein ratios. In contrast to the calorimetry data, FTIR was used primarily to study the AChR-induced perturbation of the *structure* of DEPC. Thus, changes in the FTIR spectra at a single temperature (20 °C) well above the T_m were chosen to monitor parameters such as band shape, intensity, and frequency shifts as a function of lipid to protein ratio. The vibrational spectrum was divided into three regions that correspond to the well characterized hydrocarbon, interfacial, and headgroup regions of the lipid molecule [24].

Acyl chain region. The region of the infrared spectrum between 3100 to 2800 cm^{-1} containing the carbon-hydrogen stretching fundamental vibrations has been extensively used to characterize the lipid phase state (see, for example, Ref. 24). A peak at 2854 cm^{-1}, which is assigned to the symmetrical stretching modes of the acyl chain CH_2 groups, and a peak at 2930 cm^{-1}, assigned to the CH_2 antisymmetric modes, comprise the major components of this spectral interval. Changes in frequency of the symmetric CH_2 stretching mode can be related to specific phenomena including the introduction of *gauche* conformers [25,26].

The CH_2 symmetric band is free from interference from any protein band and was used to monitor the acyl chain region of the lipid molecule. No variations in the CH_2 band frequency as a function of lipid/protein ratios were discernible. Thus, there is no indication that the protein induces changes in the *trans/gauche* ratios.

Interfacial region. The spectral region 1700–1800 cm^{-1} includes the C=O stretching mode of the lipid ester carbonyl. For pure hydrated DEPC a single asymmetric band is observed. Upon deconvolution peaks at 1740 cm^{-1} and 1721 cm^{-1} become apparent and have been traditionally assigned to the C=O modes of the *sn*-1 and *sn*-2 chains, respectively [27,28]. However, Blume et al. [29] have recently demonstrated by selective labeling of each carbonyl with ^{13}C that both carbonyls consist of two underlying components. Since these components are observed only in completely hydrated phospholipid bilayers, Blume et al. conclude that they must arise from hydration effects, i.e., hydrogen bonding of water to the carbonyl groups.

In the reconstituted samples the region from 1700 to 1760 cm^{-1} could also contain bands arising from protonated carboxyl groups of AChR side chain residues such as aspartate and glutamate [30]. While most of the carboxyl groups are expected to be ionized at pH 7.5, hydrogen/deuterium exchange can be used to distinguish the lipid ester carbonyl from the protonated carboxyl residues since carboxyl groups with exchangeable hydrogens are downshifted about 10 cm^{-1}. No difference was observed in this region upon hydrogen/deuterium exchange and all bands are assigned to the lipid carbonyls. The lipid carbonyl region was studied in reconstituted sample with increasing amounts of AChR as shown in Fig. 5. With increasing amounts of AChR and a corresponding decreasing lipid to protein mole ratio, the higher frequency band at 1742 cm^{-1} decreased in intensity while the lower frequency band at 1727 cm^{-1} increased in intensity and shifted slightly to higher frequency. These results have been compared to lipid spectra [29] and the higher frequency band in the DEPC spectrum can be assigned to non-hydrogen bonded carbonyl vibrations. Thus, the increased amount

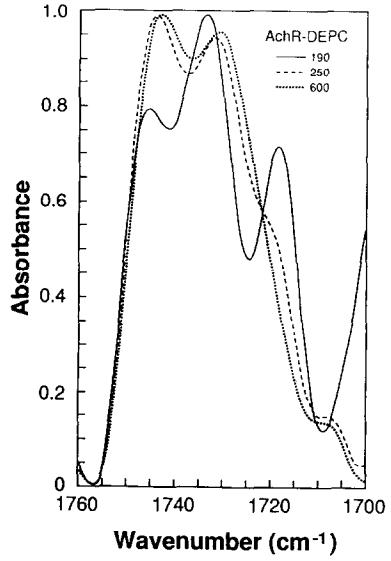

Fig. 5. Deconvoluted spectra of the lipid carbonyl stretching region of reconstituted AChR and pure DEPC membranes. Deconvolution parameters used were $\sigma = 6.0$ cm^{-1} (half-width and half-height) and $K = 2.25$ (relative reduction in bandwidth).

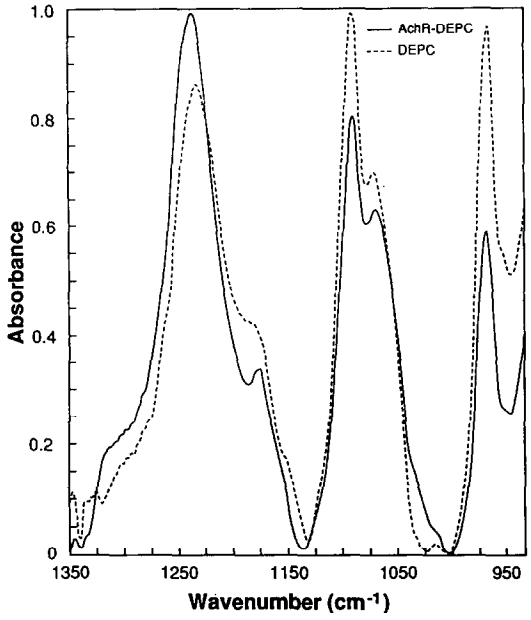

Fig. 6. FTIR absorbance spectra of the lipid headgroup region of reconstituted AChR-DEPC (solid line; lipid to protein mole ratio of 250) and pure DEPC membranes (dotted line).

of protein correlates with an apparent increase in hydrogen bonding of the carbonyl regions. Additionally, another band at 1718 cm^{-1} becomes apparent at very low lipid to protein ratios. Both of these lower frequency bands are assigned to the hydrogen bonded states of the carbonyl group.

Headgroup region. The 1350–900 cm^{-1} region of the FTIR spectrum of aqueous DEPC is shown in Fig. 6. The two absorption bands, with maxima at 1230 and 1088 cm^{-1} are assigned to asymmetric and symmetric PO_2^- stretching frequencies [31,32]. The shoulder at 1068 cm^{-1} due to the PO_2^- symmetric band is generally seen in diesterified phosphates and is assigned to the vibrational frequency of the R-O-P-O-R′ group [33]. The C-N-C asymmetric stretch appears at 977 cm^{-1} and is due to the choline headgroup moiety of DEPC [34]. The absorption bands due to the phosphatidylcholine headgroups obscure underlying protein absorption bands in this region previously identified by Fong and McNamee [7] using samples prepared in lipid mixtures containing a much lower mole fraction of phosphatidylcholine.

The 1350–900 cm^{-1} region of AChR-DEPC spectra is also shown in Fig. 6. It is evident that no significant change occurs in the presence of AChR. There is no change in frequencies or band shapes of the PO_2^- stretching, the R-O-P-O-R′ group and the C-N-C asymmetric stretch. The PO_2^- asymmetric is shifted to a higher frequency by about 5 cm^{-1}. This shift can be attributed to a slight tilt in the headgroup conformation resulting in a longer P-O bond length [35].

Discussion

The interaction of AChR with phosphatidylcholine (DEPC) leads to a small downward shift in the transition temperature, a broadening of the peak, and a reduction in the enthalpy change of the main phase transition of the lipid based on calorimetric data derived from DSC measurements. The reduction in enthalpy can be attributed to a reduction in the total number of phospholipids that undergo the transition. The decrease can be simulated in terms of a simple model in which a fixed number of lipid molecules are prevented from undergoing a cooperative phase transition. From the data we estimate that each AChR molecule removes approx. 95 DEPC molecules from the transition. This number is higher than the 45–50 molecules calculated to completely surround the perimeter of the receptor based on geometrical arguments derived from low resolution structural studies [36], but is less than the number expected for two layers of lipid around each protein molecule. A value of about 50 lipid molecules in contact with the receptor was estimated from the motionally restricted components of EPR spectra of spin-labeled reconstituted membranes [9].

The difference in the number of perturbed lipids measured by EPR and DSC for AChR may be characteristic of the nature of the lipid perturbation as viewed by the two techniques since similar differences have been observed for other integral membrane proteins [37,38]. Evidence for a secondary layer of perturbed lipids has been obtained for the reconstituted Ca^{2+}-ATPase from sarcoplasmic reticulum using a combination of calorimetric, morphological, and fluorescence assays [39]. One possible explanation is that the extracellular portion of the receptor may be perturbing some lipid molecules and removing them from the phase transition, but these lipids are not included in the motionally restricted phospholipids as determined by EPR. DSC also inherently can only detect the lipids that *cooperatively* undergo the phase transition. Thus, a number of perturbed lipids larger than the motionally restricted lipids as determined by EPR may not be surprising. An extreme case of differential lipid–protein interactions was reported by Ruppel et al. [40] using glycophorin. They found that the number of perturbed lipids depended strongly on the lipid to protein ratio and they postulated that a relaxed extracellular domain could affect about 300 lipids at high lipid to protein mole ratios and that a more structured extracellular domain could affect only 100 lipids at lower ratios where tighter packing induced the conformation change. We have seen no indication of abrupt changes in the number of perturbed lipids as the lipid to protein mole ratio is increased from 200 to 800. From the calorimetric results reported here, lipid–protein interactions appear to be short-range and AChR perturbs only the

first and perhaps part of the second shell of lipids surrounding the protein.

Functional significance for this stoichiometry of the perturbed lipid domain was suggested from experiments on delipidation of the receptor. Analysis of both ion influx activity and ligand binding showed that progressive irreversible inactivation occurred as the lipid to protein mole ratio was decreased below 45 [14]. This result is consistent with the idea of a functional requirement of a small pool of lipids that are required to maintain the receptor in a fully functional state.

FTIR spectroscopy offers complementary information on the nature of the system and permits us to focus specifically on the structural changes taking place within the bilayer. The primary infrared parameters used to study the acyl chain motion are the frequency shifts of the symmetric CH stretching mode. There is no evidence of any change in the degree of organization of the acyl chains since the trans/gauche conformer ratio of the lipids in the vicinity of the protein is constant at different lipid to protein mole ratios. However, the frequency parameter measures the average population of the trans and gauche states at equilibrium, but does not measure the rate at which they are interconverting. Motional information of the acyl chains can be extracted from the half-bandwidth of the CH_2 stretching modes. However, the observed signal arises from methylene groups along the entire length of the acyl chain causing inhomogeneous broadening of the band. Information on the dynamic nature of the interconversions could be obtained by using lipids specifically deuterated at a single position to overcome the inhomogeneous broadening problem [26].

The FTIR spectrum of DEPC shows that both carbonyl groups take part in hydrogen bonding to water. In the presence of AChR, the hydrogen bonded forms of the lipid molecules increases suggesting that incorporation of AChR increases water penetration. Carruthers and Melchior [41] have shown that water permeability in biological membranes is derived for the most part from the interaction of membrane spanning proteins with the lipid bilayer. Thus, lipid–protein interactions may govern the water and proton permeability of membranes, which have been found to be exceptionally high [42]. An alternative explanation of the increased hydrogen bonding may be a more direct interaction between the lipids and polar groups on the protein exposed at the interfacial surface. Such interactions could stabilize the structure of the protein and may account for lipid-dependent effects observed by Fong and McNamee [7].

Both the calorimetric and FTIR observations are in agreement with a motionally restricted domain observed by many spectroscopic technique [43,44,9]. The functional role of the perturbed lipid domain in acetylcholine receptor function remains to be determined. Since sterols and negatively charged lipids are known to affect both the structure and ion channel activity of the receptor [7], it is possible that these lipids create further restrictions in the motional degrees of freedom accessible to the receptor. Experiments are in progress to examine the interactions of phosphatidic acid with the receptor by FTIR techniques using the analytical approaches outlined here.

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