A Role for Cholesterol as a Structural Effector of the Nicotinic Acetylcholine Receptor†

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ABSTRACT: The effects of cholesterol on the protein structure and on the ionic channel activity of purified acetylcholine receptor (AcChR) reconstituted into lipid vesicles have been studied, respectively, by Fourier-transform infrared spectroscopy and by rapid kinetics of cation influx. Reconstitution of the AcChR in asolectin phospholipid vesicles in the absence of either cholesterol or the nonpolar lipids present in crude asolectin extracts results in a considerable loss of the ability of the AcChR to support cation channel function. This functional loss is accompanied by spectral changes in the conformationally-sensitive amide I band of the protein infrared spectrum which are indicative of alteration in the protein secondary structure. Quantitative estimation of such alteration by band-fitting analysis reveals a marked decrease in ordered protein structures such as the α-helix and β-pleated sheet, concomitant with an increase in less ordered structures appearing at 1644 cm^{-1} in the infrared spectrum. Furthermore, the addition of increasing amounts of cholesterol to the reconstituted bilayer produces a progressive, complete recovery both in the control of cation channel function and in the infrared spectrum. This restoration of AcChR structure and function by cholesterol, however, does not occur when the AcChR is reconstituted in vesicles made from purified egg phosphatidylcholine, thus suggesting that the presence in the reconstituted bilayer of phospholipids other than phosphatidylcholine may be required for cholesterol to exert its modulatory effects. Also, the increase in thermal stability of the AcChR protein observed upon addition of cholinergic agonists [Castresana, J., Fernandez-Ballester, G., Fernandez, A. M., Laynez, J. L., Arrondo, J. L. R., Ferragut, J. A., & Gonzalez-Ros, J. M. (1992) *FEBS Lett. 314*, 171–175] requires the presence of cholesterol in the reconstituted bilayer. On the other hand, as a departure from the above cholesterol dependence of structural features of the AcChR protein, we have found that the acquisition of the agonist-induced desensitized conformation by the AcChR, which is characterized by the appearance of an even more prominent structural component at 1644 cm^{-1}, is just a function of the prolonged exposure to the cholinergic agonists and does not depend upon the presence of cholesterol or any other lipid in the reconstituted bilayer. Our observations, taken along with proposals by other authors as to the existence of cholesterol binding sites on the AcChR [Jones, O. T., & McNamee, M. G. (1988) *Biochemistry 27*, 2364–2374], suggest that cholesterol may play a complex role as an allosteric effector of the AcChR at sites located on the transmembrane portion of the protein.

The nicotinic acetylcholine receptor (AcChR)[1] from *Torpedo* is a transmembrane glycoprotein composed of four different polypeptide subunits (α, β, γ, and δ) in a 2:1:1:1 stoichiometry (Jones et al., 1987; Stroud et al., 1990; Galzi et al., 1991). Binding of cholinergic agonists to sites on extracellular domains of the AcChR elicits the formation of a transient cation channel within the protein, responsible for the initiation of postsynaptic membrane depolarization. On continuous exposure to the agonist, however, the channel opening response becomes blocked and the affinity for the agonists increases, a process known as desensitization (Ochoa et al., 1989).

Studies of reconstitution of the purified AcChR protein into artificial liposomes of defined composition have shown that the presence of certain lipids in the reconstituted samples, namely, cholesterol and acidic phospholipids, is important in preserving the ability of the reconstituted AcChR to exhibit an optimal cation channel activity (Dalziel et al., 1980; Criado et al., 1982, 1984; Ochoa et al., 1983; Fong & McNamee, 1986; Jones et al., 1988; Sunshine & McNamee, 1992). In those reports, the need for cholesterol to maintain AcChR function has been studied quite extensively, partly because cholesterol is the most abundant single lipid in the native *Torpedo* membranes (Gonzalez-Ros et al., 1982). Nevertheless, in spite of the existing information on the cholesterol dependence of AcChR function, very little is known as to the molecular basis of such effects of cholesterol on the activity of an integral membrane protein. On the basis of the apparent correlation between the presence of cholesterol, modification of membrane fluidity, and alteration of AcChR function, an "optimal fluidity" hypothesis was proposed to tentatively

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[1] Abbreviations: AcChR, acetylcholine receptor; α-Bgt, α-bungarotoxin; FT-IR, Fourier-transform infrared spectroscopy; PC, phosphatidylcholine; PTSA, 1,3,6,8-pyrenetetrasulfonate; DPH, 1,6-diphenylhexa-1,3,5-triene.

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account for the effects of cholesterol on an indirect basis, that is, through modification of physical properties of the bilayer in which the AcChR is contained (Fong & McNamee, 1986). On the other hand, the possibility that cholesterol may exert its effects through direct interaction with the AcChR protein has received support from studies using spin-labeled (Ellena et al., 1983; Arias et al., 1990), brominated (Jones & McNamee, 1988), or photoactivatable (Middlemas & Raftery, 1987; Fernandez et al., 1993) steroid analogs, as well as from calorimetric studies (Artigues et al., 1989). It is conceivable, therefore, that the effects of cholesterol on AcChR activity are mediated by complex mechanisms involving more than simply membrane fluidity alterations.

Fourier-transform infrared spectroscopic (FT-IR) methods have shown great potential to detect structural differences between the various possible conformers of complex membrane proteins (Mendelsohn & Mantsch, 1986; Arrondo et al., 1987; Rothschild, 1992; Haris & Chapman, 1993), including the AcChR (Baezinger et al., 1992a,b; Görne-Tschelnokow et al., 1992; Fernandez-Ballester et al., 1992; Castresana et al., 1992; Naumann et al., 1993; Baezinger et al., 1993; Bhushan & McNamee, 1993), and owing to their flexibility to accommodate different experimental conditions, they could be of value to complement ongoing studies on the protein structure at high resolution (Unwin et al., 1988; Unwin, 1993). In this paper, we have made use of the conformational sensitivity of the amide I infrared absorbance band in the AcChR spectrum to probe the effects of cholesterol on the structure of the purified protein reconstituted into lipid vesicles. This information is presented along with data to illustrate the functional status of the reconstituted AcChR and the apparent membrane fluidity in the different reconstituted bilayers.

MATERIALS AND METHODS

Carbamylcholine chloride, deuterium oxide (D_2O, 99.9% by atom), cholesterol, and crude phosphatidylcholine-rich phospholipid extracts from soybean (type 2-S, asolectin lipids) were purchased from Sigma. Neutral (nonpolar) lipids present in the asolectin extracts were eliminated by passing the lipids, dissolved in chloroform, through a silica gel 60 (230–400 mesh, Merck) column, followed by elution with chloroform. Phospholipids in asolectin were then eluted from the column by using chloroform/methanol mixtures. Egg phosphatidylcholine (PC) (Merck) was purified to homogeneity by adsorption chromatography on an aluminum oxide column. [^{125}I]-α-Bungarotoxin (^{125}I-α-Bgt) was from New England Nuclear. The fluorophores 1,3,6,8-pyrenetetrasulfonate (PTSA) and 1,6-diphenylhexa-1,3,5-triene (DPH) were from Molecular Probes.

Acetylcholine Receptor Purification and Reconstitution. AcChR-enriched membranes were prepared from the electroplax of *Torpedo marmorata* (Artigues et al., 1987). The AcChR was purified from cholate extracts of those membranes by affinity chromatography in the presence of asolectin lipids (Jones et al., 1987). The purified AcChR had specific activities of approximately 8 nmol of α-Bgt bound/mg of protein.

Plain lipid vesicles were prepared from whole asolectin lipids, asolectin phospholipids, or egg PC, at ~40 mg/mL, with or without added cholesterol at different molar ratios, by a detergent dialysis procedure (Castresana et al., 1992; Fernandez et al., 1993). The resulting lipid vesicles were resolubilized in 4% sodium cholate and used immediately for reconstitution.

Reconstituted AcChR samples were prepared by mixing aliquots of purified AcChR with the solubilized lipid vesicles from above. Final concentrations in the reconstitution mixtures were the following: AcChR, ~1 mg/mL; phospholipids, ~5 mg/mL; sodium cholate, 1.5%; cholesterol, molar percentages ranging 0 to 40%, with respect to the phospholipids. Under these conditions, the phospholipid to protein molar ratio ranged from 2000 to 3000. Reconstitution was accomplished by dialysis at 4 °C for about 50 h (6 × 1 L changes in 10 mM Tris, pH 7.4, containing 100 mM NaCl, followed by 2 × 1 L changes in 10 mM Hepes, pH 7.4, and 100 mM NO_3Na). Protein, phospholipid (lipid phosphorus), and cholesterol concentrations were determined as previously described (Escriba et al., 1990).

Infrared Measurements. Exchange of water by D_2O in the reconstituted AcChR samples was carried out by submitting the samples to at least two centrifugation–resuspension cycles in D_2O buffers of identical saline composition as the original H_2O media (Castresana et al., 1992). The samples for FT-IR analysis, at a concentration of ~20 mg of protein/mL, were placed into a liquid demountable cell (Harrick Ossining, NY) equipped with CaF_2 windows and 50-μm-thick mylar spacers. FT-IR spectra were taken in a Nicolet 520 instrument equipped with a DTGS detector, as previously described (Fernandez-Ballester et al., 1992). Fourier derivation and deconvolution of the spectra were carried out as reported by others (Moffatt & Mantsch, 1992). Derivation was performed using a power of 3, breakpoint of 0.3. Deconvolution was performed by using a Lorenztian bandwidth of 18 cm^{-1}, a resolution enhancement factor of 2.25, and a Bessel apodization.

For quantitative estimation of protein secondary structure, the original amide I band was decomposed into its constituents by a curve-fitting analysis similar to the one described earlier (Castresana et al., 1992), except that the spectral base line was corrected prior to starting the fitting procedure. Component bands included in the band decomposition routine were always those detected upon band-narrowing of the spectra. The initial estimates of the height of each component for the iterative fitting process were set at 90% of the absorbance, measured in the original spectra at each of their frequency maxima, for the bands in the wings and for the most intense component, and at 70% of the original absorbance for all the other bands (Arrondo et al., 1993). The goodness of the fitting procedure was tested by generating artificial spectra from individual components and vice versa.

AcChR Functional Measurements. Agonist-mediated cation translocation through the reconstituted AcChR was monitored by using a "stopped-flow/fluorescence quenching" assay of Tl^+ influx (Gonzalez-Ros et al., 1984). The assay is based on the rapid collisional quenching of the fluorescence of 1,3,6,8-pyrenetetrasulfonate (PTSA) entrapped into the reconstituted AcChR vesicles, by externally added Tl^+ (Fernandez et al., 1993). For convenience, the reconstituted AcChR samples used for the Tl^+ influx assays were prepared at phospholipid:protein ratios double than those indicated above, since it increases the efficiency of PTSA entrapment into the vesicles.

Fluorescence Polarization Measurements. Steady-state fluorescence polarization experiments were performed as described previously (Canaves et al., 1991), using DPH as the fluorophore and a double-channel SLM-8000 C spectrofluorometer with Glan-Thompson calcite prism polarizers in the excitation and emission beams. Plain lipid vesicles corresponding to all the different lipid mixtures used in this study were prepared identically to the reconstituted AcChR vesicles, but in the absence of AcChR protein. Temperature was controlled by a water bath circulator connected to the

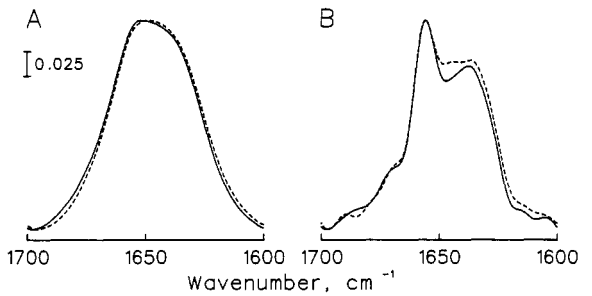

FIGURE 1: Infrared amide I band region of the original (A) and deconvolved (B) spectra of AcChR reconstituted in lipid vesicles made from whole asolectin (control, solid line) and from neutral lipid-depleted asolectin phospholipids (dashed trace). Reconstituted AcChR vesicles in H_2O media [10 mM Hepes (pH 7.4)/100 mM $NaNO_3$] were submitted to isotopic exchange as indicated under Materials and Methods. The D_2O media used to resuspended the membrane samples had the same saline composition as that of the original H_2O media. In this and in all the other figures, the spectrum of D_2O was subtracted from those of the membrane-containing samples. The spectra were recorded at 20 °C. The bar in panel A represents 0.025 absorbance unit.

fluorometer cuvette holder. The concentration of lipid vesicles was 20 μM, in terms of lipid phosphorus, and the molar ratio of DPH to phospholipid was 1:500. Samples were excited at 360 nm, and the emission of DPH fluorescence was monitored by using Corning 3-74 cutoff filters to eliminate scattered light.

RESULTS

The main features of the 1800–1500-cm^{-1} region in the infrared spectra of purified AcChR reconstituted into vesicles made of whole asolectin lipids have been described previously (Castresana et al., 1992). The strong amide I band, comprising the 1600–1700-cm^{-1} spectral region (Figure 1A), results primarily from stretching vibrations of C=O groups in peptide bonds (Arrondo et al., 1993), the exact frequencies of which depend on the nature of the hydrogen bonding involving the C=O groups which, in turn, is determined by the particular secondary structure adopted by the protein. Resolution-enhancement, band-narrowing techniques (Mantsch et al., 1988; Moffatt & Mantsch, 1992), such as Fourier self-deconvolution or Fourier derivation, show that the amide I region exhibits maxima at approximately 1605, 1615, 1636, 1656, 1672, 1680, and 1690 cm^{-1} (Figure 1B). Whereas the 1605- and 1615-cm^{-1} components correspond to amino acid side-chain vibration, all the other maxima have been assigned to vibration of the carbonyl group in peptide bonds within different AcChR secondary structural motifs (Fernandez-Ballester et al., 1992; Castresana et al., 1992): the 1636-cm^{-1} band is assigned to β-sheet structure, the 1656-cm^{-1} band to α-helix, the 1690- and 1680-cm^{-1} bands to turns, and the 1672-cm^{-1} band includes contributions from turns as well as from the $(0,\pi)$ β-sheet vibration band.

Figure 1 also shows that reconstituting in the absence of neutral lipids, i.e., in purified asolectin phospholipids, which results in a pronounced decrease in the ability of the AcChR to allow cation translocation in response to binding of cholinergic agonists (Table 1), results also in alteration of the spectral shape of the amide I band (Figure 1A). Deconvolution of the amide I band narrows the underlying spectral components and shows that the main changes produced in the absence of neutral lipids refer to an alteration in the 1656: 1636-cm^{-1} absorbance ratio, also seen in the original spectra, and to the appearance of a prominent shoulder centered at approximately 1642 cm^{-1}, which is not detected in the deconvolved spectra corresponding to AcChR reconstituted in whole asolectin (Figure 1B). Other spectral changes can also be seen at the 1700–1670-cm^{-1} region.

Table 1: Stopped-Flow Kinetics of Carbamylcholine-Activated Tl^+ Influx in Reconstituted AcChR Vesicles Made from Different Lipid Mixtures[a]

lipid matrix	k_{app}^{max} (s^{-1})	K_D ($M \times 10^4$)
whole asolectin lipids	131 ± 15	5.16 ± 0.57
phospholipids from asolectin	59 ± 16	4.33 ± 0.42
+10% cholesterol	65	3.14
+20% cholesterol	105	4.37
+40% cholesterol	132 ± 34	7.58 ± 0.22
egg phosphatidylcholine	NA[b]	NA
+40% cholesterol	NA	NA

[a] Reconstituted vesicles in 10 mM Hepes buffer (pH 7.4)/200 mM $NaNO_3$ were rapidly mixed with an equal volume of 10 mM Hepes buffer, pH 7.4, 170 mM $NaNO_3$, and 30 mM $TlNO_3$ containing increasing concentrations of carbamylcholine, ranging from 0 to 250 μM (final concentration), in a Hi-Tech SF-51 stopped-flow instrument. Influx of Tl^+ into the reconstituted AcChR vesicles was recorded as a time-dependent quenching of the fluorescence of PTSA entrapped into the vesicles (see Materials and Methods). The apparent maximum rate constant for Tl^+ influx (k_{app}^{max}) and the dissociation constant (K_D) were determined as in Gonzalez-Ros et al. (1984). [b] No activity.

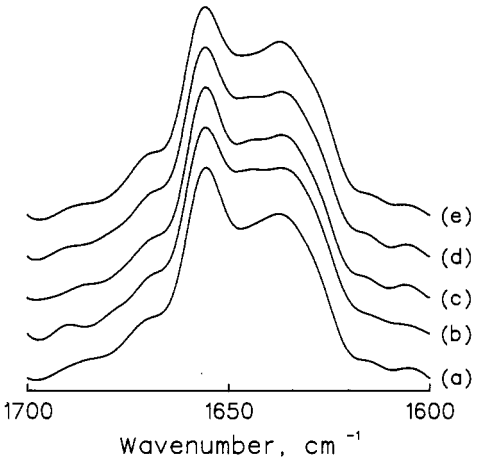

FIGURE 2: Deconvolved amide I infrared band of AcChR samples reconstituted in whole asolectin (a), in neutral lipid-depleted asolectin (asolectin phospholipids) (b), and in neutral lipid-depleted asolectin containing added cholesterol at 10 (c), 20 (d), and 40 (e) molar percentages. In this and in all the other figures, the molar percentages of cholesterol are given with respect to the phospholipids.

Reconstitution of the purified AcChR in the presence of increasing molar percentages of cholesterol in cholesterol/asolectin phospholipid mixtures, which results in the progressive recovery of the AcChR ion channel activity (Table 1), results also in the progressive restoration of the infrared spectral shape which, at a cholesterol concentration (~40%) resembling that found in the native *Torpedo* electric tissue (Gonzalez-Ros et al., 1982), becomes similar to that observed when using whole asolectin lipids as the reconstitution matrix (Figure 2).

Quantitative estimation of the protein secondary structural changes responsible for the observed alterations in spectral shape requires the application of methods based either on curve-fitting of the spectra or on the use of calibration sets [for a discussion on quantification methods, see Arrondo et al. (1993)]. On the former, it should be noted that the determination of the protein secondary structure in absolute terms from the infrared spectra remains a controversial issue subjected to potential sources of error as diverse as (i) the inevitable subjectivity in the definition of the spectral base line or of the initial guesses used in the iterative fitting process

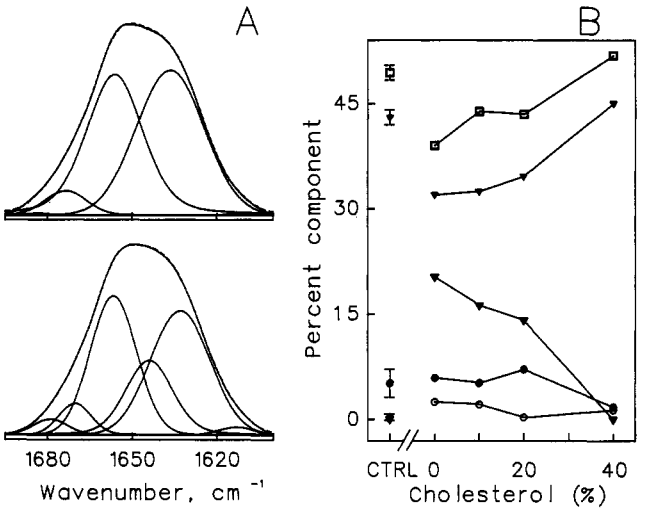

FIGURE 3: Band-fitting analysis of the infrared amide I band of AcChR reconstituted in lipid vesicles of different composition. Panel A show representative results from the band-fitting analysis of AcChR secondary structure. The main components of the amide I band of AcChR reconstituted in whole asolectin (upper traces) and in neutral lipid-depleted asolectin (lower traces) are shown. The discontinuous trace, superimposed on the original spectra, represents the theoretical curve resulting from the contribution of all individual components. Panel B shows the estimated percentages of the individual components at 1681 (open circles), 1672 (closed circles), 1656 (closed triangles), 1644 (open triangles), and 1636 cm^{-1} (open squares) for AcChR reconstituted in whole asolectin (CTRL, control) and for the other samples indicated in the legend to Figure 2.

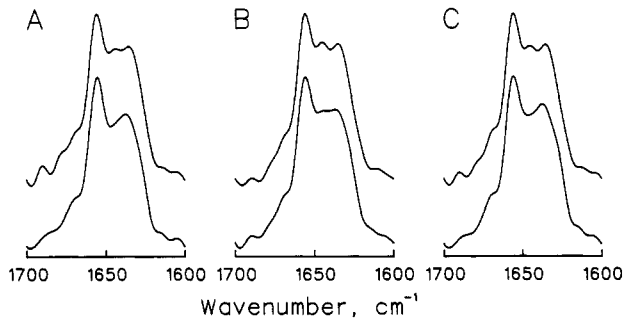

FIGURE 4: Effects of carbamylcholine on the deconvolved infrared amide I band of AcChR reconstituted in whole asolectin lipid vesicles (A), in neutral lipid-depleted asolectin (B), and in neutral lipid-depleted asolectin supplemented with 40 molar percent cholesterol (C). Upper and lower traces in the figure represent, respectively, samples in the presence and in the absence of 10^{-2} M carbamylcholine.

or (ii) the not necessarily unique solution to satisfy the complex mathematical functions used in the fitting of the spectral data (Surewicz et al., 1993). Therefore, to obtain reliable fittings, restrictions have to be applied to the possible solutions to avoid artifacts (Arrondo et al., 1993) (see Materials and Methods). Figure 3A illustrates the type of results that can be obtained from the band-fitting analysis of the original amide I spectral band used in this work. Figure 3B summarizes such results and shows that reconstitution of the AcChR in the absence of neutral lipids produces a pronounced decrease in the percentages of both 1636-cm^{-1} and 1656-cm^{-1} bands, which are assigned to β-sheet and α-helical structures, respectively, concomitant with the increase in a component band centered at 1644 cm^{-1} which accounts for more than 20% of the protein secondary structure. Figure 3B also shows that, similar to the observations made on the original spectra, increasing the concentration of cholesterol in the reconstituted matrix containing the purified asolectin phospholipids causes a progressive restoration of the different secondary structural

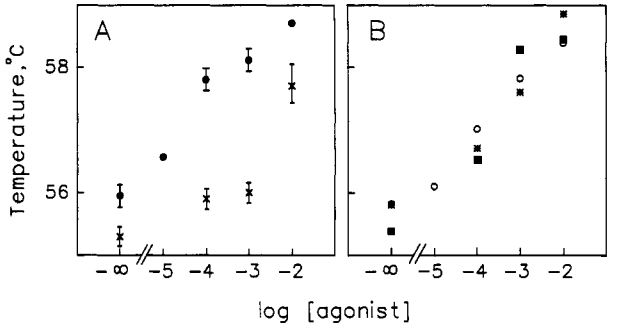

FIGURE 5: Effects of carbamylcholine on the thermal stability of reconstituted as determined by monitoring the temperature-dependence of the infrared amide I band. Protein denaturation temperatures (on the y axes) were determined as in Fernandez-Ballester et al. (1992). Panels A and B show the concentration dependence of the stabilizing effect observed with carbamylcholine in AcChR samples reconstituted in whole asolectin (closed circles), in neutral lipid-depleted asolectin (crosses), and in neutral lipid-depleted asolectin supplemented with cholesterol at 10 (asterisks), 20 (open circles), and 40 (closed squares) molar percent.

components to percentages similar to those observed initially in the samples reconstituted in whole asolectin.

The interaction of cholinergic agonists at high concentration with the reconstituted AcChR has been previously shown to have certain effects on the infrared spectra, which were attributed to AcChR "structural desensitization" (Castresana et al., 1992). Figure 4 shows that the agonist-induced spectral changes reported previously (Castresana et al., 1992) occur also in all samples studied here and do not depend on whether or not cholesterol or the neutral lipids from asolectin are present in the reconstituted membrane. In fact, at the concentration of agonist used in these studies (10^{-2} M), the spectra of the AcChR reconstituted in whole asolectin or in neutral lipid-depleted asolectin, with or without added cholesterol at high concentration, are very similar (Figure 4, upper traces), despite the fact that the spectra of those samples recorded in the absence of ligand are quite different from each other (Figure 4, lower traces).

In our previous report on the effects of cholinergic agonists on AcChR reconstituted in whole asolectin, it was also shown that the receptor thermal denaturation temperature increases when the agonist concentration is increased, thus suggesting concentration-dependent changes in the protein conformation below the denaturation temperature (Castresana et al., 1992). Figure 5A shows that such an agonist-induced thermal stabilization phenomenon is strongly diminished when the AcChR protein is reconstituted in the absence of neutral lipids but it is easily regained when cholesterol is present in the reconstituted matrix, even at the lowest cholesterol concentration used in these studies (Figure 5B).

The effects of cholesterol on AcChR structure and function described above, using asolectin phospholipid/cholesterol mixtures to reconstitute the AcChR, have also been assessed using egg PC/cholesterol mixtures as the reconstitution matrix. In the latter matrix, regardless of the presence of cholesterol at concentrations similar to those used above, the reconstituted AcChR does not exhibit ion channel activity (Table 1), in consonance with previous reports by other authors (Ochoa et al., 1983; Fong & McNamee, 1986; Sunshine & McNamee, 1992). Figure 6A shows that the amide I infrared band of AcChR reconstituted in egg PC vesicles differs from that observed in AcChR reconstituted in whole asolectin. Moreover, the infrared spectrum of the egg PC-based samples does not seem to be substantially altered by the presence or the absence of cholesterol in the reconstituted mixture (Figure

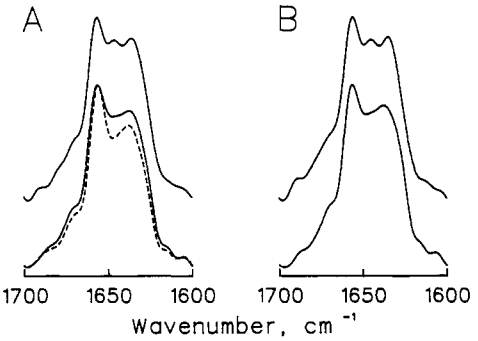

FIGURE 6: Deconvolved infrared amide I band of AcChR samples reconstituted in lipid vesicles made from egg PC (A) and from egg PC supplemented with cholesterol at 40 molar percent (B), in the absence (lower traces) and in the presence (upper traces) of carbamylcholine. The deconvolved spectrum of AcChR reconstituted in whole asolectin (dashed trace), in the absence of agonist, has been included in panel A to facilitate comparison with the egg PC-based samples.

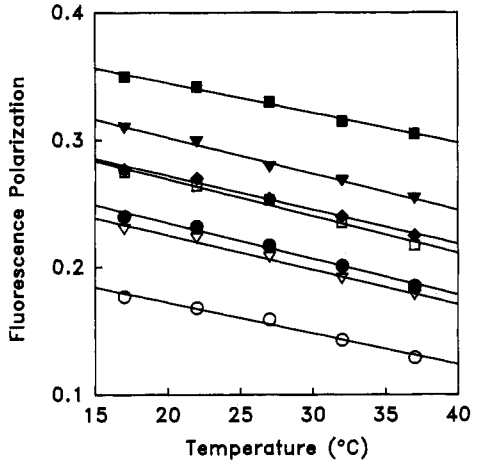

FIGURE 7: Effect of cholesterol on the temperature dependence of the steady-state fluorescence polarization of DPH in large unilamellar egg PC (open symbols) and in neutral lipid-depleted asolectin (filled symbols) vesicles containing 0 (circles), 10 (diamonds), 20 (triangles), and 40 (squares) molar percent cholesterol.

6B). On the other hand, we observed that the addition of cholinergic agonists to these samples to produce desensitization (Figure 6, upper traces) induces an alteration of the infrared spectral shape comparable to that found upon desensitization of the asolectin reconstituted samples. This seems consistent with observations made by others (Criado et al., 1982; Ochoa et al., 1983; Fong & McNamee, 1986; Jones et al., 1988; Sunshine & McNamee, 1992) that the AcChR reconstituted in purified PC vesicles still retains the ability to undergo agonist-induced sensitization–desensitization transitions.

Figure 7 shows that, regardless of whether asolectin phospholipids or egg PC is used as the reconstitution matrix, the addition of increasing amounts of cholesterol to the bilayer has the expected effects on decreasing the apparent fluidity of the resulting reconstituted membranes, as determined from the temperature-dependent fluorescence depolarization of the hydrophobic probe DPH. It is also observed that at all temperatures studied, there is overlapping between the fluorescence polarization values exhibited by samples in which the AcChR has no ion channel activity whatsoever (the egg PC-based samples) and those exhibited by moderately active samples (the asolectin phospholipid samples containing intermediate concentrations of cholesterol).

DISCUSSION

On the basis of the conformational sensitivity of the infrared amide I band of the protein spectrum, different FT-IR approaches are currently used by several laboratories in an attempt to provide structural informational on the AcChR. The main findings reported so far could be grouped as it follows: First, differences in the protein structure have been observed between the resting and the agonist-induced desensitized conformations of the AcChR, both in AcChR-rich native membranes from *Torpedo* (Fernandez-Ballester et al., 1992; Baezinger et al., 1992b; Görne-Tschelnokow et al., 1992) and in reconstituted lipid vesicles containing purified AcChR as the sole protein component (Castresana et al., 1992; Baezinger et al., 1992a, 1993). Second, the protein secondary structure has been determined from curve-fitting of the original (Castresana et al., 1992) or the deconvolved amide I band (Naumann et al., 1993) which, in spite of the existing differences between the type of AcChR samples and the band-decomposition methods used, predicts a predominance of β-sheet structure in the AcChR protein, in apparent agreement with current diffraction data (Unwin, 1993). Third, the thermal denaturation of the AcChR monitored by FT-IR (Fernandez-Ballester et al., 1992; Castresana et al., 1992; Naumann et al., 1993) exhibits denaturation temperatures similar to those determined by calorimetry (Castresana et al., 1992) and includes the loss of ordered protein secondary structures, such as the α-helix and β-sheet, and the appearance of less ordered components at $\sim$1620, 1640, and 1684 cm^{-1}.

In this report, we have used the protein's infrared amide I band in an attempt to provide an explanation for the known effects of cholesterol in modulating the ion channel function of reconstituted AcChR, based upon the modification of the AcChR protein structure by cholesterol. A previous attempt to use infrared methods to study the effects of cholesterol on AcChR structure was based on monitoring infrared bands in the so-called skeletal region (ranging from 1000 to 900 cm^{-1}) of the protein spectrum (Fong & McNamee, 1987) and yielded uncertain results, partly because of the low intensity of the spectroscopic signal and the weakly established correlation of the selected vibrational modes to specific protein structures.

The studies reported here, using the infrared amide I band of reconstituted AcChR, strongly suggest that the presence of cholesterol within the reconstituted bilayer mediates major changes in the spectral shape and in the estimated secondary structure of the purified AcChR protein in a concentration-dependent manner. These protein structural changes, which occur correlative to the cholesterol-mediated regaining of the ability of the AcChR to control its characteristic cation gating activity, refer mainly to the stabilization by cholesterol of ordered protein structures such as the α-helix and β-sheet, while minimizing the appearance of a component band at 1644 cm^{-1}. In spectra taken in D_2O, the 1644-cm^{-1} component in the infrared amide I band is usually assigned to nonordered protein structures and is related to the band shifting to approximately 1657 cm^{-1}, overlapping with the α-helix, when the spectra are taken in H_2O (Arrondo et al., 1993). Other authors (Fabian et al., 1992), however, have recently described a component band in the spectrum of streptokinase in aqueous media at approximately 1640 cm^{-1} which does not shift in D_2O and has been assigned to "flexible polypeptide loops". In our case, it is possible that, as seen in other proteins (Arrondo et al., unpublished results), the observed 1644-cm^{-1} component in the AcChR infrared spectra comprises contributions from both structural motifs from above. In any case, a spectral component at approximately 1642 cm^{-1} was also observed as

a consequence of "loosening" the native β-sheet structure of the AcChR by desensitization induced by cholinergic agonist (Castresana et al., 1992).

As different from the observations made in the asolectin phospholipid matrix, when the AcChR is reconstituted into egg PC vesicles, the addition of cholesterol results in (i) no recovery of the AcChR cation gating activity and (ii) minimal spectral changes in the infrared amide I band. These observations indicate that phospholipids other than PC may be required for cholesterol to exert its functional and structural effects on the AcChR protein. From the functional viewpoint, it is well-known that acidic phospholipids such as phosphatidic acid, cardiolipin, or others are required along with cholesterol to allow optimal control of AcChR cation gating (Dalziel et al., 1980; Ochoa et al., 1983; Criado et al., 1984; Fong & McNamee, 1986; Sunshine & McNamee, 1992) and thus it is possible that those phospholipids could also be required to enable cholesterol to act as a structural modulator of the AcChR. In relation to this, it has been recently shown by infrared spectroscopic methods that phosphatidic acid interacts quite strongly with the AcChR and serves itself as a protein structural effector (Bhushan & McNamee, 1993).

More evidence on the occurrence of structural alteration of the AcChR by cholesterol has been obtained from thermal denaturation experiments. In these experiments, infrared spectra are taken at increasingly higher temperatures to produce denaturation of the protein samples, which can be monitored either by difference spectroscopy (Yang et al., 1987; Naumann et al., 1993) or by measuring the bandwidth or the frequency maxima of spectral components in the amide I band (Fernandez-Ballester et al., 1992; Muga et al., 1993). The temperature at which the thermal event is produced is indicative of protein packing, and changes in its value have been related to conformational modifications in the nondenatured protein involving differences in secondary (Arrondo et al., 1988) or in tertiary structure (Muga et al., 1993). We have already reported that the presence of cholinergic agonist stabilizes the AcChR protein against thermal denaturation both in native membranes from *Torpedo* (Fernandez-Ballester et al., 1992) and in reconstituted vesicles made from whole asolectin lipids (Castresana et al., 1992). In this work, we show that reconstitution in the absence of neutral lipids prevents quite efficiently the agonist-induced increase in protein thermal stability and that such a feature can be fully regained by the addition of cholesterol to the reconstituted bilayer, even at the lowest cholesterol concentration used in these studies. These observations further suggest an increasingly complex role for cholesterol as a modulator of AcChR structure.

The experiments carried out in the presence of high concentrations of carbamylcholine provide additional information that cholesterol is not required for the agonist to produce the structural changes leading to AcChR desensitization. Such desensitization-related structural changes have been estimated already from curve-fitting of the infrared amide I band (Castresana et al., 1992) and by difference spectroscopy (Baezinger et al., 1992a, 1993). The former revealed concentration-dependent spectral changes occurring at agonist concentrations ranging from 10^{-5} to 10^{-3} M, which refer mainly to a progressive decrease in a β-sheet spectral component at 1633 cm^{-1} and an increase in a less ordered component at 1642 cm^{-1}. On the other hand, Baezinger et al. (1992a, 1993), using films of AcChR vesicles deposited on the surface of a germanium reflection element and difference spectroscopy at 50 μM agonist, also showed changes in the protein amide I spectral region where positive peaks were found at 1655 and ~1640 cm^{-1} and a negative one at 1620 cm^{-1}. We report here that, regardless of whether the protein is reconstituted in asolectin or egg PC, and in the presence or in the absence of cholesterol, the infrared amide I bands of the desensitized protein in the different reconstituted media are always very similar. Our interpretation, therefore, is that the acquisition of the desensitized conformation depends ultimately just upon the presence of the cholinergic agonist, independently of how perturbed the protein might be as a consequence of reconstitution in different lipid environments, prior to addition of the ligand.

The question remains as to the nature of the molecular events that could possibly be responsible for the observed effects of cholesterol in modulating AcChR structure and function. An earlier proposal by McNamee's group (Fong & McNamee, 1986) suggested the possibility that cholesterol regulation of membrane fluidity constitutes the key factor in determining the effects of cholesterol on the AcChR. We have found, however, that there are samples of AcChR reconstituted in egg PC/cholesterol mixtures, with no ion translocation activity, which exhibit apparent fluidity values identical to those corresponding to active samples reconstituted in asolectin/cholesterol mixtures. Therefore, we conclude that the alteration of membrane fluidity by cholesterol could perhaps contribute to some extent to the observed effects of cholesterol on AcChR structure and function but it seems far from being the determining factor in these phenomena. On the other hand, there is strong experimental evidence to support that cholesterol interacts directly with the AcChR protein. For instance, on the basis of fluorescence quenching studies, a finite number of relatively specific cholesterol binding sites (estimated as 5–10 sites per AcChR molecule) have been postulated on the transmembrane region of the AcChR protein (Jones & McNamee, 1988). Also, it has been shown that the AcChR can be easily labeled by a photoactivatable cholesterol analogue (Fernandez et al., 1993) and that such photolabeling (i) can be displaced by unlabeled cholesterol and (ii) is sensitive to the protein structural rearrangement induced by desensitization. These observations lead us to believe that there could be a truly specific interaction between cholesterol and defined sites on the AcChR protein and that such an interaction could be responsible for the effects of cholesterol on AcChR structure and function reported here. This implies that, besides its role as a bilayer constituent, cholesterol may act as an "allosteric effector" on regulatory sites at the lipid-protein interface of the AcChR. Such putative interaction seems complex, and its occurrence requires at least the presence in the bilayer of phospholipids other than PC as discussed above. As to the nature and location of such cholesterol binding sites, a proposal has been already made on a merely theoretical basis (Ortells et al., 1992) which assumes the contribution of α-helical portions of the M1, M3, and M4 transmembrane domains of the AcChR to form the cholesterol binding pockets. Such proposal, however, does not consider recent findings on the structure of AcChR at 9-Å resolution (Unwin, 1993) which show that the transmembrane portion of the protein is predominantly formed by β-pleated sheets. Finally, since there are other integral membrane proteins whose activities are known to depend upon the presence of cholesterol (Whetton et al., 1983; Whetton & Houslay, 1983; Vemuri & Philipson, 1989; Bolotina et al., 1989), it seems possible that a similar "allosteric" modulation of the protein structure by cholesterol may be implicated in the functional regulation of such molecules.

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REFERENCES

Arias, H. R., Sankaram, M. B., Marsh, D., & Barrantes, F. J. (1990) *Biochim. Biophys. Acta 1027*, 287–294.

Arrondo, J. L. R., Mantsch, H. H., Mullner, N., Pikula, S., & Martonosi, A. (1987) *J. Biol. Chem. 262*, 9037–9043.

Arrondo, J. L. R., Young, N. M., & Mantsch, H. H. (1988) *Biochim. Biophys. Acta 952*, 261–268.

Arrondo, J. L. R., Muga, A., Castresana, J., & Goñi, F. M. (1993) *Prog. Biophys. Mol. Biol. 59*, 23–56.

Artigues, A., Villar, M. T., Ferragut, J. A., & Gonzalez-Ros, J. M. (1987) *Arch. Biochem. Biophys. 258*, 33–41.

Artigues, A., Villar, M. T., Fernandez, A. M., Ferragut, J. A., & Gonzalez-Ros, J. M. (1989) *Biochim. Biophys. Acta 985*, 325–330.

Baezinger, J. E., Miller, K. W., McCarthy, M. P., & Rothschild, K. J. (1992a) *Biophys. J. 62*, 64–66.

Baezinger, J. E., Miller, K. W., & Rothschild, K. J. (1992b) *Biophys. J. 61*, 983–992.

Baezinger, J. E., Miller, K. W., & Rothschild, K. J. (1993) *Biochemistry 32*, 5448–5454.

Bhushan, A., & McNamee, M. G. (1993) *Biophys. J. 64*, 716–723.

Bolotina, V. M., Omelyanenko, V., Heyes, B., Ryan, U., & Bregestovsky, P. (1989) *Eur. J. Physiol. 415*, 262–268.

Canaves, J. M., Ferragut, J. A., & Gonzalez-Ros, J. M. (1991) *Biochem. J. 279*, 413–418.

Castresana, J., Fernandez-Ballester, G., Fernandez, A. M., Laynez, J. L., Arrondo, J. L. R., Ferragut, J. A., & Gonzalez-Ros, J. M. (1992) *FEBS Lett. 314*, 171–175.

Criado, M., Eibl, H., & Barrantes, F. J. (1982) *Biochemistry 21*, 3622–3629.

Criado, M., Eibl, H., & Barrantes, F. J. (1984) *J. Biol. Chem. 259*, 9188–9198.

Dalziel, A. W., Rollins, E. S., & McNamee, M. G. (1980) *FEBS Lett. 122*, 193–196.

Ellena, J. F., Blazing, M. A., & McNamee, M. G. (1983) *Biochemistry 22*, 5523–5535.

Escriba, P. V., Ferrer-Montiel, A. V., Ferragut, J. A., & Gonzalez-Ros, J. M. (1990) *Biochemistry 29*, 7275–7282.

Fabian, H., Naumann, D., Misselwitz, R., Ristau, O., Gerlach, D., & Welfle, H. (1992) *Biochemistry 31*, 6532–6538.

Fernandez-Ballester, G., Castresana, J., Arrondo, J. L. R., Ferragut, J. A., & Gonzalez-Ros, J. M. (1992) *Biochem. J. 288*, 421–426.

Fernandez, A. M., Fernandez-Ballester, G., Ferragut, J. A., & Gonzalez-Ros, J. M. (1993) *Biochim. Biophys. Acta 1149*, 135–144.

Fong, T. M., & McNamee, M. G. (1986) *Biochemistry 25*, 830–840.

Fong, T. M., & McNamee, M. G. (1987) *Biochemistry 26*, 3871–3880.

Galzi, J. L., Revah, F., Bessis, A., & Changeux, J. P. (1991) *Annu. Rev. Pharmacol. 31*, 37–72.

Gonzalez-Ros, J. M., Paraschos, A., Llanillo, M., & Martinez-Carrion, M. (1982) *Biochemistry 21*, 3467–3474.

Gonzalez-Ros, J. M., Ferragut, J. A., & Martinez-Carrion, M. (1984) *Biochem. Biophys. Res. Commun. 120*, 368–375.

Görne-Tschelnokow, U., Hucho, F., Naumann, D., Barth, A., & Mäntele, W. (1992) *FEBS Lett. 309*, 213–217.

Haris, P. J., & Chapman, D. (1993) *Biochem. Soc. Trans. 21*, 9–15.

Jones, O. T., & McNamee, M. G. (1988) *Biochemistry 27*, 2364–2374.

Jones, O. T., Earnest, J. P., & McNamee, M. G. (1987) in *Biological Membranes. A Practical Approach* (Findlay, J. B. C., & Evans, W. H., Eds.) pp 139–178, IRL Press, Oxford.

Mantsch, H. H., Moffatt, D. J., & Casal, H. L. (1988) *J. Mol. Struct. 173*, 285–298.

Mendelsohn, R., & Mantsch, H. H. (1986) in *Progress in Protein–Lipid Interactions* (Watts, A., & DePont, A., Eds.) pp 103–146, Elsevier, Amsterdam.

Middlemans, D. S., & Raftery, M. A. (1987) *Biochemistry 26*, 1219–1223.

Moffatt, D. J., & Mantsch, H. H. (1992) *Methods Enzymol. 210*, 192–200.

Muga, A., Arrondo, J. L. R., Bellon, T., Sancho, J., & Bernabeu, C. (1993) *Arch. Biochem. Biophys. 300*, 451–457.

Naumann, D., Schultz, C., Görne-Tschelnokow, U., & Hucho, F. (1993) *Biochemistry 32*, 3162–3168.

Ochoa, E. L. M., Dalziel, A. W., & McNamee, M. G. (1983) *Biochim. Biophys. Acta 727*, 151–162.

Ochoa, E. L. M., Chattopadhyay, A., & McNamee, M. G. (1989) *Cell. Mol. Neurobiol. 9*, 141–178.

Ortells, M. O., Cockcroft, V. B., Lunt, G. G., Marsh, D., & Barrantes, F. J. (1992) in *Membrane Proteins: Structures, Interactions and Models* (Pullman, A., Ed.) pp 185–198, Kluwer Academic, Dordrecht, The Netherlands.

Rothschild, K. J. (1992) *J. Bioenerg. Biomemb. 24*, 147–167.

Stroud, R. M., McCarthy, M. P., & Shuster, M. (1990) *Biochemistry 29*, 11009–11023.

Sunshine, C., & McNamee, M. G. (1992) *Biochim. Biophys. Acta 1108*, 240–246.

Surewicz, W. K., Mantsch, H. H., & Chapman, D. (1993) *Biochemistry 32*, 389–394.

Unwin, N. (1993) *J. Mol. Biol. 229*, 1101–1124.

Unwin, N., Toyoshima, C., & Kubalek, E. (1988) *J. Cell Biol. 107*, 1123–1138.

Vemuri, R., & Philipson, K. D. (1989) *J. Biol. Chem. 264*, 8680–8685.

Whetton, A. D., & Houslay, M. D. (1983) *FEBS Lett. 157*, 70–74.

Whetton, A. D., Gordon, L. M., & Houslay, M. D. (1983) *Biochem. J. 212*, 331–338.

Yang, P. W., Mantsch, H. H., Arrondo, J. L. R., Saint-Girons, I., Guillou, Y., Cohen, G. N., & Barzu, O. (1987) *Biochemistry 26*, 2706–2711.