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A Raman spectroscopic study of acetylcholine receptor-rich membranes from *Torpedo marmorata*. Interaction of the receptor with carbamylcholine and (+)-tubocurarine

Dimitrina Aslanian [a], Pal Grof [a,1], Jean-Luc Galzi [b] and Jean-Pierre Changeux [b]

[a] *Laboratoire de Physique des Solides, Associé au CNRS, Université Pierre et Marie Curie, Paris (France) and*
[b] *Laboratoire de Neurobiologie Moléculaire, Associé au CNRS, Institut Pasteur, Paris (France)*

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Raman spectroscopy is used to determine structural features of alkali-treated subsynaptic membrane fragments from *Torpedo marmorata* electric organ, rich in native functional AcChR. Distinct vibrations attributable to the membrane proteins and lipids were identified and studied before and after addition of the agonist carbamylcholine and the competitive antagonist (+)-tubocurarine. The protein secondary structure determined by using amide-I polypeptide vibrational analysis, indicates 47% α-helices, 25% β-sheets, 18% turns and 11% undefined structure. The secondary structure of the AcChR molecule was not subject to large modifications upon addition of carbamylcholine. But, the presence of the (+)-tubocurarine leads to detectable changes in the amide-I region which might be interpreted as reflecting different contributions of α-helices and turns in the secondary structure. In addition, Raman spectra provide information about the environment of aromatic amino acids (tyrosine and tryptophan), the (C-C) bonds, the CH_2 and CH_3 groups of aliphatic side chains, as well as the disulfide (S-S) and cystein (C-S) bonds. The tyrosines seem 'exposed' to the aqueous medium. The Raman spectra of the AcChR-carbamylcholine complex suggest 'exposed' tryptophans, while those of the unliganded membrane-bound AcChR or of the receptor with (+)-tubocurarine are shown 'buried'. The disulfide bridges in the AcChR subunits show identical conformation in the absence and presence of carbamylcholine. On the contrary, considerable changes are found in the AcChR-(+)-tubocurarine complex. Carbamylcholine and especially (+)-tubocurarine decrease lipid fluidity.

Introduction

In an approach to the molecular organisation of the subsynaptic membrane and of its main components, we have undertaken Raman spectroscopic studies on membrane fragments rich in acetylcholine receptor (AcChR) purified from *Torpedo marmorata* electric organ, and on some of their macromolecular and lipidic components [1,2]. These studies have illustrated the utility of laser Raman spectroscopy in obtaining information on the conformation of the proteins and lipids which compose the subsynaptic membrane.

The nicotinic AcChR from fish electric organ, or at the neuromuscular junction, is a ligand-gated ion channel composed of four classes of subunits arranged in an heterologous $\alpha_2\beta\gamma\delta$ transmembrane pentamer [3–6]. It contains the binding sites for cholinergic ligands, an ion channel selective for cations, and all the elements that ensure the coupling between them.

The agonists, like carbamylcholine, activate ion flux through the channel upon binding to two sites per receptor molecule. Upon prolonged exposure to agonist, the receptor undergoes conformational transitions towards a non responsive desensitized state [7].

Competitive antagonists, such as (+)-tubocurarine (curare), bind to the same sites as agonists but inhibit their action. Some of them stabilize, at equilibrium, a desensitized conformation of the receptor [8,9].

Electron microscopy studies of the AcChR have provided information on the general shape and dimensions of the molecule [10,11], the approximate distribution of the molecule across the membrane [12,13] and the relative positions of the subunit chains [4,14]. The amino-acid sequences of all subunits from *Torpedo* receptor have been determined [15–17]. On the bases

Correspondence to: D. Aslanian, Laboratoire de Physique des Solides, Associé au CNRS, Université Pierre et Marie Curie, 4 place Jussieu, 75251 Paris, France.

[1] Permanent address: Institute of Biophysics, Semmelweis Medical University, Budapest, Hungary.

of these sequence data, analyses of hydropathy plots, and empirical secondary structure predictions [4,18,19], models for AcChR structure have been proposed [20]. In general, these models incorporate at least four transmembrane α-helices per subunit, named M-I to M-IV, of which M-II is proposed to form the ion channel [5,6].

Although evidence is now accumulating in favour of the helical organization of at least some of the transmembrane segments [21,22], and the folding of α-subunit around the cholinergic ligand-binding area, little is known about the actual three-dimensional structure of the extramembraneous domains.

In the present work, we use Raman scattering to refine the structural analysis of subsynaptic membrane fragments from *T. marmorata* electric organ, containing native functional AcChR in the absence or presence of the agonist carbamylcholine or the competitive antagonist (+)-tubocurarine.

Evidence is presented, that when AcChR protein is exposed to carbamylcholine its secondary structure does not undergo considerable changes, whereas in the presence of (+)-tubocurarine modifications of this structure can be detected. Changes on the conformation of some side chains such as disulfide bridges, methionine residues, (C-C) groups, as well as of the lipid components occur upon action of carbamylcholine and (+)-tubocurarine.

Materials and Methods

Biological materials

AcChR-rich membrane fragments were prepared from *T. marmorata* electric organ after Saitoh et al. [23] and alkali-treated as described [24]. The specific binding activity was typically 4–5 nmol of α-bungarotoxin sites per mg of proteins and the functionality of the receptor in the membrane preparation was systematically checked by determining dansyl-C_6-choline binding kinetics according to Heidmann et al. [25].

For Raman spectroscopy, membrane fragments were pelleted three times from the desired buffer, the supernatant was discarded and membranes were homogenized at a concentration of 8–10 mg protein/ml. The composition of the buffer for spectroscopy was 10 mM Tris-HCl (pH 7.5), 3 mM EDTA and 1 mM EGTA.

Commercial carbamylcholine and (+)-tubocurarine (Sigma) were recrystallized twice before use. Their Raman spectra recorded at a concentration of 1 mM do not exhibit any peaks in the investigated regions.

Carbamylcholine or (+)-tubocurarine were added at a final concentration of 1 mM to occupy more than 95% of the available binding sites (80 μM).

Lipid extracts were obtained as described [26]. Dried extracts were resuspended in Tris buffer and sonicated.

Raman spectroscopy

Capillary tubes (1 mm inner diameter, 10 μl samples) were held in vertical position in the middle of a specially constructed brass cell thermostated at 10°C.

Raman measurements were performed with a Jobin-Yvon Ramanor U 1000 double monochromator. The samples were excited with an argon laser (Coherent Innova 90–3) tuned at 514.5 nm. Excitation power was about 130 mW at the sample level. The spectral slits were 500/320 nm.

Data evaluation and spectrum analysis

The digitally recorded spectra (usually 30 scans were collected for the same sample) were averaged, buffer and fluorescence contributions were subtracted. The spectra were smoothed by Fourier transforms.

In a given batch of *Torpedo* electric organs, we obtained a highly reproducible sample quality which yielded a set of Raman spectra with the standard frequency deviations (± 2 cm^{-1} for the strong bands and ± 4 cm^{-1} for the weak ones) described in Raman spectroscopic studies. Between different batches of *Torpedo* electric organ, we sometimes observed the presence of variable amounts of carotenoids, the vibrations of which are situated near 1160 and 1525 cm^{-1}. No other detectable sources of variability were observed.

The secondary structure of the proteins were quantitatively determined by the method of Williams [27,28]. In this method, the solvent spectrum and aromatic-ring side-chain vibrations are first subtracted from the amide-I region. The amide-I spectrum is then fitted with a linear combination of amide-I spectra of standard proteins whose structure is known from X-ray diffraction. The contribution of the various types of secondary structures are deduced from the fitting coefficients. The average error of the secondary structure determination is $\pm 5\%$ [27,28].

The region of amide-I bands of the proteins studied in this work contains contributions of lipids which also had to be subtractad. Lipid contributions are subtracted as follows: Difference spectra of AcChR-rich membrane fragments and of a lipid extract were generated. Criteria for subtraction were that the signal intensity of carotenoid vibration near 1525 cm^{-1} should approximate zero, and the spectral intensity in the other regions cannot be negative.

Spectra of lipid extract and of proteins were normalized at 1525 cm^{-1} to the same amplitude, and different subtraction factors were applied to find the best fit of the above two criteria. During the establishment of the evaluation procedure, different values were used to test the errors coming from the incomplete subtraction. The test indicated, that even a change of the subtraction factor from 1.0 to 0.6, i.e., an about 40% change

causes smaller errors than the error limit of the secondary structure determination (±5%).

For resolution enhancement the method of modified Jansson-VanCittert [29] constrained iterative deconvolution scheme was applied. This technique has the advantage, in comparison with that of the self-deconvolution, that the non-negativity and uniqueness of the true spectrum can be assured by the constraints as it was developed and applied previously to image enhancement problems and Raman spectra by Agard at al. [30,31].

Deconvolution of amide-I region has also the advantage, that the ring vibrations of the aromatic amino-acid residues could simply be eliminated. That is, lines coming from these vibrations should be deleted from the deconvoluted spectrum. Reconvolution of this spectrum with the shape and width used for resolution-enhancement results in the spectrum of amide-I band, free from other vibrations. These spectra were used for secondary-structure determination. For resolution enhancement in the amide-I region we used a Gaussian-Lorentzian product function of equal width with 11.5 cm^{-1} half-width. The same lineshape was used in the 2800–3100 cm^{-1} region but with 12 cm^{-1} half-width. The resolution enhancement was stopped when the difference between the original and reconvoluted spectra was smaller than 5%. To avoid overdeconvolution, the resolution-enhancement procedure was always begun with smaller linewidth which ensures absence of artifacts in the main part of the spectra. The resolution-enhanced spectra with greater linewidth were compared to the spectra with smaller linewidth, and those which seemed to be overdeconvoluted were rejected.

Results

Fig. 1A shows the 400 to 1800 cm^{-1} region of AcChR-rich membrane fragments after subtraction of the contributions of buffer and fluorescence background. Smoothing was achieved by transformed Fourier. Assignments of frequencies are given. Fig. 1B displays the spectrum of AcChR-rich membrane fragments in the presence of the agonist carbamylcholine. The spectrum of membrane fragments with AcChR exposed to the competitive antagonist (+)-tubocurarine is given in Fig. 1C. Some distinct vibrations

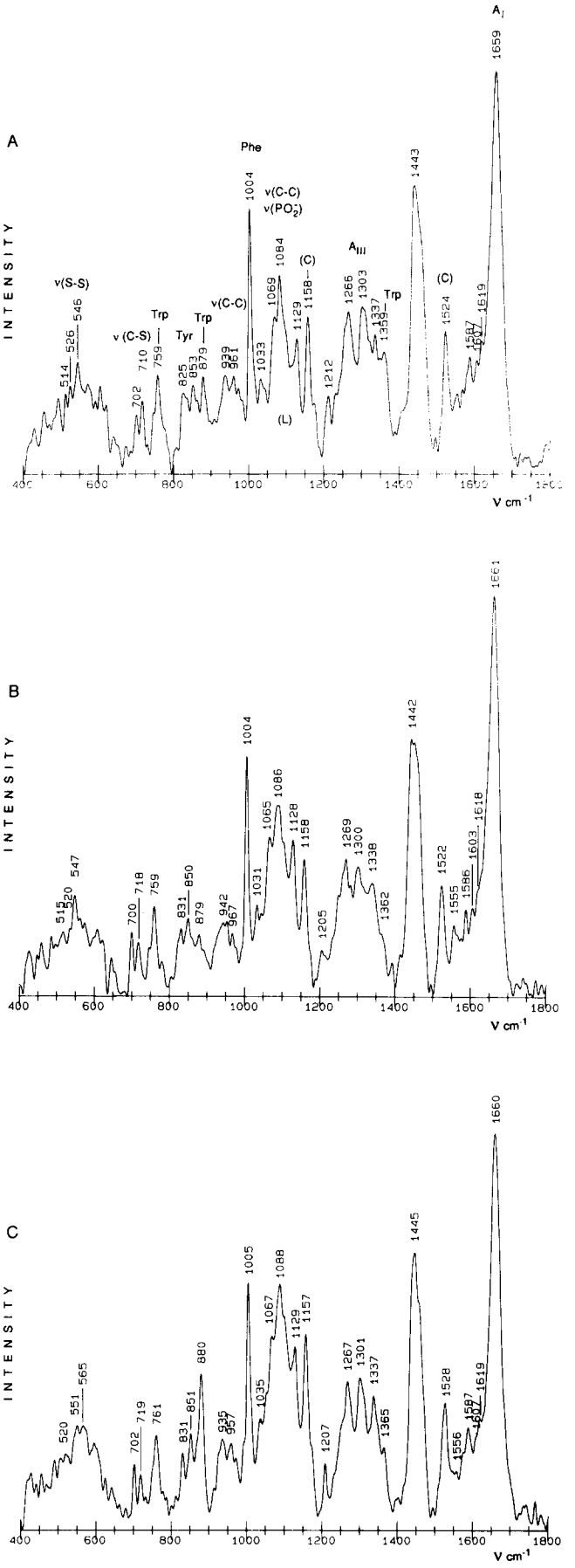

Fig. 1. (A) Raman spectrum of AcChR-rich membrane fragments from *T. marmorata* in 10 mM Tris-HCl (pH 7.5), 3 mM EDTA, 1 mM EGTA, after water and fluorescence subtraction. (B) Raman spectrum of AcChR-rich membrane fragments from *T. marmorata* in the presence of the agonist carbamylcholine (recording conditions as in A). (C) Raman spectrum of AcChR-rich membrane fragments from *T. marmorata* with AcChR exposed to the competitive antagonist (+)-tubocurarine (recording conditions as in A).

attributable to the membrane proteins and lipids can be identified. Most of them are assignable to specific types of protein and lipid groups. Thus, the present results focus on the assigned protein and lipid vibrations, as well as on the modifications of these vibrations after addition of carbamylcholine or (+)-tubocurarine.

Protein vibrations

Fig. 1A–C shows Raman vibrations permitting to obtain informations on the secondary structure of the proteins, as well as on the side-chain environments which include the aromatic amino acids (tyrosine and tryptophan), and aliphatic side chains (S-S, C-S, C-C, C-N, CH_2 and CH_3).

Secondary structure estimation

Alkali-treated postsynaptic membrane fragments contain AcChR molecules and other non-receptor proteins. In the samples of AcChR-rich membrane fragments, the AcChR molecule is preponderant as shown in Fig. 2. However, traces of remaining non-receptor proteins in the gel patterns are also visible. Thus, we must take into account that the estimation of secondary structure of AcChR protein will be, to some extent, modified by the presence of non-receptor protein molecules.

Generally, the amide-I region is used for the evaluation of the secondary structure of proteins. The Raman spectra of AcChR-rich membrane fragments (Fig. 1A–C) disclose a sharp amide-I vibration centered near 1660 cm^{-1}.

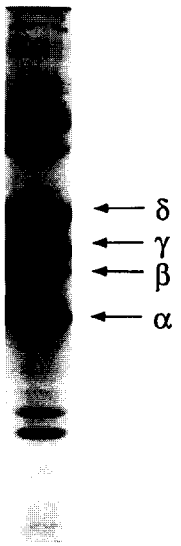

Fig. 2. SDS-polyacrylamide gel electrophoresis of alkali-treated AcChR-rich membrane fragments. 30 μg of proteins were electrophoresed on a 10% acrylamide gel and stained with Coomassie blue. The four bands corresponding to the α, β, γ and δ-subunits are indicated.

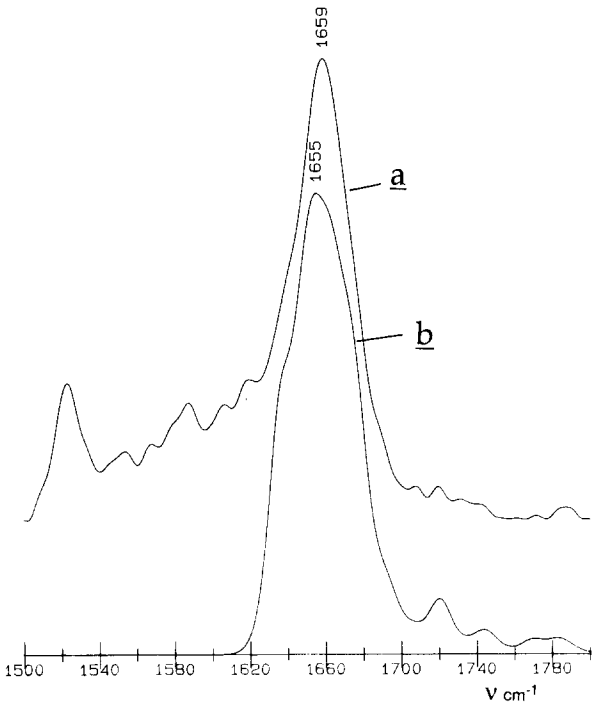

Fig. 3. The amide-I region of Raman spectrum of AcChR-rich membrane fragments from *T. marmorata* (a) after subtraction of lipid vibrations, (b) after subtraction of aromatic amino-acid ring vibrations.

Quantitative assessment of secondary structure, using amide-I band, is complicated by the ν(C = C)-stretching vibrations of the membrane lipids which overlap this band. We recorded the Raman spectrum of the lipid extract from AcChR-rich membranes (data not shown). According to this spectrum, the ν(C = C)-stretching vibrations of membrane lipids have been located at 1661 cm^{-1}. This spectrum was used later when we subtracted the lipid contributions from amide-I band. The amide-I band of proteins arises from peptide group vibrations which are sensitive to local perturbations of the environment. The shape and frequency of the band are influenced by the relative contributions of different secondary structures. Determination of secondary structures from the amide-I band in a multi-component system requires subtraction of all other bands which are not due to the protein in question. After subtraction of lipids, several features still remained because of the ring vibrations of the aromatic amino-acid side chains (Fig. 3a). These were also deleted (see Materials and Methods) and the remaining curve (Fig. 3b) was used for quantitative estimation of the AcChR secondary structure by the method described in Refs. 27 and 28.

As shown in Table I, the membrane fragments in 10 mM Tris buffer, with AcChR in native state, contain on average 47% total helical structure (α_t: 29% ordered and 18% disordered helices) and about 25% total β-sheets (β_t: 24% antiparallel and 1% parallel).

TABLE I

Secondary structure estimation of the AcChR-rich membrane fragments of T. marmorata electric organ

	Structure (%)			
	α_t (α_o α_d)	β_t (β_{ap} β_p)	T	U
Native membrane-bound AcChR	47 (29 18)	25 (24 1)	18	11
AcChR-carbamylcholine complex	48 (35 13)	25 (24 1)	18	10
AcChR-(+)-tubocurarine complex	39 (21 17)	26 (20 6)	25	13

Further, we found about 18% reverse turns (T) and 11% undefined structure (U).

After addition of carbamylcholine the secondary structure determination yields 48% total helical structure (35% ordered and 13% disordered), 25% β-sheets (24% antiparallel and 1% parallel), 18% reverse turns (T) and 10% undefined structure (U). In the presence of (+)-tubocurarine the secondary structure is composed of 39% helices (21% ordered and 17% disordered), 26% β-sheets (20% antiparallel and 6% parallel), 25% turns and 13% undefined structure.

Characteristic side-chain vibrations

I. Aromatic amino-acid ring vibrations

Tyrosine ring. The characteristic Raman peaks of the well-known tyrosine doublet at 850–830 cm^{-1} appear in the spectrum of membrane fragments with native AcChR at 853 and 825 cm^{-1} (Fig. 1A). Yu et al. [32] reported that the relative intensity of this doublet changes depending on the local environment. They correlated the intensity ratio of these two vibrations (I_{850}/I_{830}) with tyrosine residues in two different environments: 'exposed' to hydrophilic regions and 'buried' within the protein in hydrophobic regions. It was shown [33] that the ratio is 2.5 for 'exposed' tyrosines (i.e., the phenol ring acts as a strong H-bond acceptor); it is 0.3 for 'buried' tyrosines (i.e., the phenol ring acts as a strong H-bond donor). The ratio is 0.9–1.3 when tyrosine residues are 'normally' exposed, i.e., involved in moderate H-bonds.

In the Raman spectra of AcChR-rich membrane fragments, the intensity ratios of tyrosine doublets indicated the following values: $I_{853}/I_{825} = 1.1$ (native AcChR, Fig. 1A); $I_{850}/I_{831} = 1.15$ (AcChR with carbamylcholine, Fig. 1B) and $I_{851}/I_{831} = 1.25$ (AcChR with (+)-tubocurarine, Fig. 1C). These ratios suggest that the tyrosine residues mostly reside in hydrophylic regions, likely they are majoritarily 'exposed' to the aqueous medium.

Tryptophan residues. It was shown [34,35] that the peak near 1360 cm^{-1} is sensitive to the tryptophan 'buried'/'exposed' state. When the vibration appears as a sharp peak this indicates that the indole ring is 'buried'. As the ring becomes accessible to water molecules, the intensity of the peak decreases.

The tryptophan residues in the membrane fragments with native AcChR (Fig. 1A) and AcChR after addition of (+)-tubocurarine (Fig. 1C) seem to be partly 'buried' and partly 'exposed' because the vibration at 1359 cm^{-1} appears in both spectra as a well separated but not very strong peak. The same vibration is shown as a weak shoulder in the spectrum of AcChR interacting with carbamylcholine (Fig. 1B), indicating that in this case the tryptophan residues are most probably on the surface of the protein molecule 'exposed' to aqueous medium.

Another important tryptophan vibration is situated near 880 cm^{-1} in the spectra of AcChR-rich membrane fragments. Kitagawa et al. [36] pointed out that the intensity of this vibration can also serve as a probe for the environment of tryptophan residues in a protein molecule. Using the ratio I_{880}/I_{1450} they indicated that this ratio diminishes when tryptophan residues become 'exposed'. For the native AcChR we found the ratio $I_{879}/I_{1443} = 0.36$, whereas for AcChR-carbamylcholine and AcChR-(+)-tubocurarine complexes this ratios were $I_{879}/I_{1442} = 0.24$ and $I_{880}/I_{1445} = 0.57$, respectively. The lower value (0.24) for AcChR-carbamylcholine complex confirms that in this case the tryptophan residues are 'exposed' to the aqueous medium. However, tryptophans seem to be much more 'buried' in AcChR-(+)-tubocurarine complex than in the case of the native AcChR.

II. Aliphatic side-chain vibrations

(S-S)-stretching vibrations. The (S-S)-stretching vibrations appear around 510, 525 and 540 cm^{-1}. Raman spectra of a great number of model compounds indicate that the 510 cm^{-1} vibration corresponds to a *gauche-gauche-gauche* configuration of the C-C-S-S-C-C linkage; the 525 cm^{-1} to the *trans-gauche-gauche* and the 540 cm^{-1} frequency to the *trans-gauche-trans* [37].

These vibrations were found in the spectrum of the membrane fragments with native AcChR at 514, 526 and 546 cm^{-1} (Fig. 1A). They do not change significantly when AcChR interacts with carbamylcholine (Fig. 1B). However, when AcChR interacts with (+)-tubocurarine, there is a definite broadening and the frequencies increase to 520, 551 and 565 cm^{-1} (Fig. 1C) which could be interpreted as the result of conformational changes in the local geometry of the disulfide linkages.

(C-S)-stretching vibrations. The frequencies and relative intensities of the (C-S)-stretching vibrations of methionine residues depend on their conformation [34]. These vibrations appear near 700 cm^{-1}. The methionine side group, $-CH_2CH_2SCH_2$, can adopt different molecular conformations in solution. Three peaks at 650, 675 and 723 cm^{-1} are considered to be characteristic of this group [38]. The last band is attributed to

the *trans* form, the 675 cm^{-1} vibration to the *gauche* form and the 650 cm^{-1} vibration to both forms. When methionine has been incorporated into a peptide chain such as Gly-L-Met-Glu, the analogous lines appear at 655, 700 and 724 cm^{-1} [39].

In the spectrum of membrane fragments with native AcChR no line is observed at 655 cm^{-1}. A well-pronounced doublet at 702 and 710 cm^{-1} suggests the presence of the *gauche* (702 cm^{-1}) and *trans* (710 cm^{-1}) forms. Both vibrations undergo changes in intensities and frequencies when either carbamylcholine or (+)-tubocurarine were bound to the receptor. This suggests that the state of the (C-S) cross-links are different in the native AcChR and in the AcChR-carbamylcholine or in the AcChR-(+)-tubocurarine complexes.

Skeletal (C-C) and (C-N)-stretching vibrations (900–1150 cm^{-1}). As reported for viral proteins [40], the pair of lines near 940 and 960 cm^{-1} is correlated with the total valine plus leucine content. In our spectra the doublet appears as follows: in the native AcChR (Fig. 1A) it is at 939 and 961 cm^{-1}; in the AcChR-carbamylcholine complex (Fig. 1B) it is situated at 942 and 967 cm^{-1}; we found it in the AcChR-(+)-tubocurarine complex (Fig. 1C) at 935 and 957 cm^{-1}. The variation of both frequencies in the three spectra suggests that the exposition and environment of the (C-C) and (C-N) bonds of these amino-acid residues are not similar.

In the region 1000–1150 cm^{-1} the protein spectra give three vibrations near 1057 cm^{-1} (lysine, glutamine, serine), 1103 cm^{-1} (alanine) [40] and 1120 cm^{-1} which are most probably ν(C-C) mode related to the optical skeletal vibrations of polymethylenes [41]. Since we found the same vibrations as weak bands in the spectra of pure acetylcholinesterase protein molecules without lipids [42], we attributed the very strong bands which appear in the AcChR-rich membrane fragments, centered near 1085 cm^{-1}, as predominant lipid vibrations and discuss them below in the section 'Lipid vibration'.

(C-H)-bending vibrations (1300–1340 cm^{-1}). The vibrations of this region have been attributed to the CH_2 wagging, twisting and rocking motions [43]. The frequency and intensity are sensitive to *gauche-trans* isomerisations, chain branching and substitutions. The 1338 cm^{-1} vibration has been specifically and quantitatively correlated to the number of *gauche* configurations at the penultimate carbon of an otherwise *trans* chain [41,44,45]. This vibration appears as a strong signal in the spectrum of AcChR-(+)-tubocurarine complex (Fig. 1C), but its intensity is lower in the remaining two spectra (Fig. 1A and 1B), suggesting reduction of the number of *gauche* configurations in the native AcChR and in the receptor interacting with carbamylcholine.

(C-H)-stretching vibrations (2800–3120 cm^{-1}). The

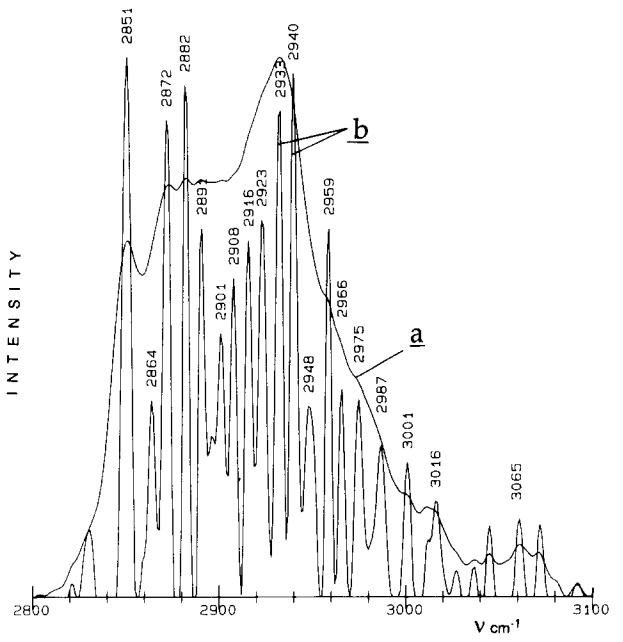

Fig. 4. Raman spectra of AcChR-rich membrane fragments from *T. marmorata* in the (C-H)-stretching region (recording conditions as in Fig. 1A): (a) experimental spectrum; (b) deconvoluted spectrum.

experimental spectra of this region (Fig. 4a) present quite similar large bands with a maximum near 2934 cm^{-1}. Two shoulders are seen in the left slopes. The first shoulder possesses a vibration at 2851 cm^{-1}. The second one is very large (between 2860 and 2930 cm^{-1}) and suggests the presence of numerous not well-resolved vibrations. To resolve them we used the procedure of constrained iterative deconvolution. Deconvolution generally provides a convenient and powerful alternative to improving experimental resolution of intrinsically broad and overlapping bands in Raman spectra [40]. An example of the deconvoluted spectra is given in Fig. 4b. The Raman lines in this spectral region can originate from (C-H)-stretching vibrations of the membrane protein or lipid molecules. To distinguish between these two possibilities, we recorded the Raman spectrum of bovine serum albumin in 10 mM Tris buffer (Fig. 5a,b) which contains a very high helical content (71%, our estimate). When comparing both experimental and deconvoluted spectra (Fig. 4a,b and Fig. 5a,b), it becomes clear that the vibration at 2851 cm^{-1} belongs to the lipids. This vibration was previously assigned to the symmetric CH_2-stretching vibrations of phospholipids [46]. The vibration at 2872 cm^{-1} (Fig. 4b) was not observed with liposomes [47] or with solutions of phosphatides in organic solvents, but it appears in the spectrum of albumin (Fig. 5a,b), as well as in spectra of variety of amino acids, polypeptides and proteins [48–50]. We therefore assign the shoulder at 2872 cm^{-1} (Fig. 4a,b) to hydrocarbon residues in the amino-acid side chains of membrane proteins.

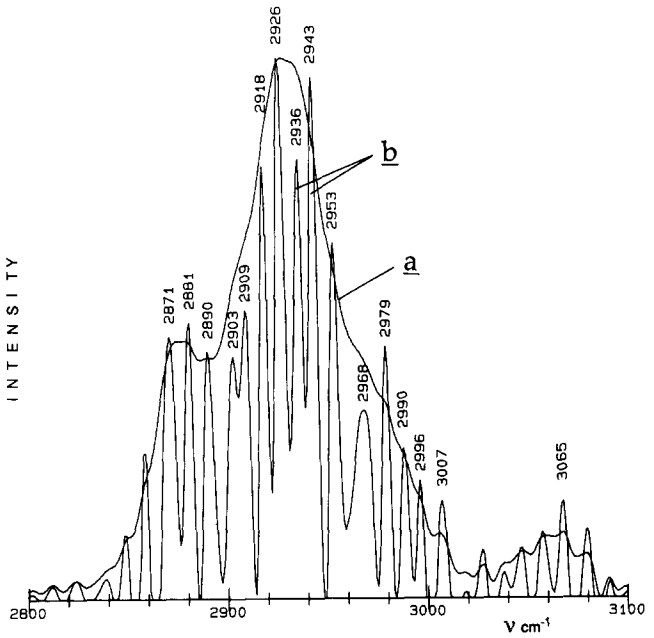

Fig. 5. Raman spectra of bovin serum albumin in 10 mM Tris-HCl (pH 7.0) after water and fluorescence subtraction: (a) experimental spectrum; (b) deconvoluted spectrum.

It is well-known that the long-chain lipids show two strong bands at 2850 and 2880 cm^{-1} and a weak large band near 2930 cm^{-1} [51–53]. Both strong lipid vibrations are of particular interest in the study of the state of lipids in AcChR-rich membrane fragments. As mentioned above, the 2851 cm^{-1} lipid vibration appears separate from the protein vibrations. This is not the case with the 2880 cm^{-1} vibration. We found a vibration at the same frequency (2881 cm^{-1}) in the spectrum of the albumin (Fig. 5b). Evidently, the protein vibration which is superimposed with the lipid vibration could easily influence the intensity of the latter.

The band near 2940 cm^{-1} (Fig. 4a) also includes contributions from both proteins and lipids. Proteins show strong scattering in this region, i.e., 2926, 2936 and 2943 cm^{-1} for albumin (Fig. 5b). The lipid contribution near 2930 cm^{-1} [53] being very weak, we suggest that the Raman scattering near 2930 cm^{-1} arises from methylene asymmetric ($\nu_a CH_2$) and methyl symmetric ($\nu_s CH_3$) vibrations of the amino-acid side chains.

The frequencies situated between 2891 and 2920 cm^{-1} have been tentatively assigned to Fermi resonance-enhanced overtones of the protein methyl deformations observed as a large shoulder near 1450–1469 cm^{-1} (Fig. 1A–C) [54–56]. The vibrations between 2958 and 2990 cm^{-1} probably correspond to the asymmetric stretching modes of different protein methyl groups. The highest frequencies at 3012 and 3065 cm^{-1} have been attributed to the asymmetric stretching vibrations of the NH_3^+ groups ($\nu_a NH_3^+$) [38].

Percentage contributions of different protein vibrations were deduced from the deconvoluted spectra (Fig. 4a). We would like to emphasize that when deconvolution is used for resolution enhancement instead of peak intensities one should use the surfaces of the resolved vibration, because the invariant quantity is the surface and not the intensity in the deconvolution. In Fig. 6 we compare the differences of the percentage

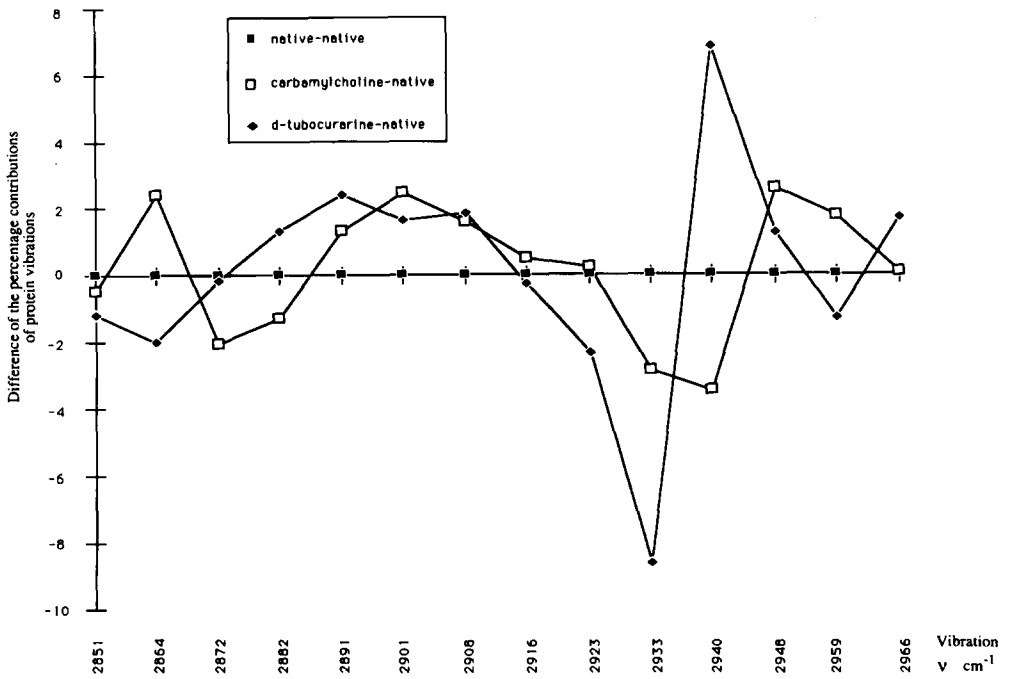

Fig. 6. Percentage contributions of different protein vibrations in the spectral range 2800–3120 cm^{-1} deduced from the deconvoluted spectrum in Fig. 4b.

contributions in this region for the membrane fragments with native AcChR with those of carbamylcholine-and (+)-tubocurarine-treated AcChR complexes. The differences of contributions were calculated as the contribution of a given vibration minus that of the native AcChR. The average of the overall differences is about $\pm 2\%$. The greatest differences are obtained for the frequencies at about 2930 and 2940 cm^{-1} which were assigned as asymmetric CH_2 and symmetric CH_3-stretching vibrations.

Lipid vibrations

Raman spectrum of membrane phospholipids monitors the vibrations from both head groups and the long hydrocarbon tail. There is evidence, that the width and ordering of the tail region of a bilayer plays an important role in the stability of a membrane.

As mentioned above, there are two regions which characterize the state of lipids: (1) the skeletal (C-C)-stretching region between 1050 and 1150 cm^{-1}, which includes also the symmetric stretching vibrations of the phosphate head group (PO_2^-) and, (2) the (C-H)-stretching region (2800–3000 cm^{-1}).

Vibrations in the 1050–1150 cm^{-1} region are sensitive to the conformational state of lipid hydrocarbon chains. Numerous studies have been performed by Raman scattering to understand the prominent vibrations of lipid molecules. There are three characteristic lipid vibrations: two of them – situated at 1130 cm^{-1} and 1064 cm^{-1} – have been assigned to the skeletal optical mode of all-*trans* conformers and, the third one – which appears near 1090 cm^{-1} – was assigned to the specific vibrations of the *gauche* structures [57]. Weak contribution to the 1070–1080 cm^{-1} region from underlying symmetric PO_2^--stretching modes are relatively unimportant, in comparison to contributions of the (C-C)-stretching modes in this spectral region [58]. The vibrations at 1130 and 1064 cm^{-1} appear as intense peaks in the crystalline state of lipids, but decrease in the disordered liquid-crystal state, where an intense band at 1090 cm^{-1} predominates. These phenomena have been attributed to the decrease in amount of all-*trans* crystal structures and introduction of structures containing *gauche* configurations in the disordered chains.

The Raman spectrum of the membrane fragments with native AcChR (Fig. 1A) shows a very strong signal at 1084 cm^{-1} and two shoulders at 1069 and 1129 cm^{-1}. The relative intensities indicate a disordered state of the hydrophobic chains of lipids, associated to the receptor molecule. As shown in Fig. 1B and C, the interaction of the AcChR with carbamylcholine or (+)-tubocurarine seems not to affect the lipid-hydrocarbon peaks in the region 1050–1150 cm^{-1}. However, a subtle feature observed in spectra indicates a small frequency shift of the most prominent vibration from 1084 cm^{-1} obtained in the membrane fragments with native AcChR (Fig. 1A) to 1086 cm^{-1} (Fig. 1B, AcChR-carbamylcholine) and 1088 cm^{-1} (Fig. 1C, AcChR-(+)-tubocurarine). At the same time the whole band becomes more intense. This shift is thought to result from a decrease in the amount of *gauche* (C-C) conformers [59], thus decreasing the lipid fluidity in the presence of carbamylcholine and (+)-tubocurarine. We found a confirmation of these observations in examining the second spectral region, characteristic for the lipid state (2800–3000 cm^{-1}).

The inter- and intra-molecular disordering processes in lipid bilayers can also be monitored through changes in the relative peak heights of 2850 and 2880 cm^{-1} vibrations. These features are assigned to methylene symmetric stretching ($\nu_s CH_2$) and methylene asymmetric stretching ($\nu_a CH_2$) modes [60]. It has been shown [46,61] that the I_{2880}/I_{2859} ratio can serve as a measure of lipid-chain mobility.

As discussed above, in our experimental spectra (Fig. 4a,b) the lipid vibration at 2851 cm^{-1} is better resolved than the second vibration near 2880 cm^{-1}. The neighbouring vibrations mainly due to the protein molecules could easily modify the intensity of the 2880 cm^{-1} vibration. Thus, when we tried to estimate the lipid fluidity using both regions (i.e., from vibrations near 1100 cm^{-1} and from the ratio I_{2880}/I_{2850}), a discrepancy was found between the two estimates.

In order to resolve this discrepancy, we recorded the spectrum of the lipid extract and plotted this spectrum together with the experimental spectrum of the membrane fragments with native AcChR. As shown in Fig. 7a and b, the 2880 cm^{-1} vibration appears in the lipid extract spectrum much less intense than the 2849 cm^{-1} vibration (Fig. 7b). The ratio I_{2880}/I_{2850} is low, which happens when lipid chains are in a disordered conformation. This is in complete agreement with the conclusion drawn when we dicussed the lipid features in the 1050–1150 cm^{-1} region. The resolution enhanced spectra of this region for the different AcChR corroborate our image. We found the same situation in the deconvoluted spectra (Fig. 4b). We used these spectra to make a quantitative estimation of the ratio I_{2880}/I_{2850}. When one uses the deconvoluted spectra instead of the intensity ratios, the ratios of the area under a peak should be taken. We found the following values: 0.53 (for the native AcChR), 0.69 (for AcChR interacting with carbamylcholine) and 0.77 (for AcChR interacting with (+)-tubocurarine). Increasing values indicate that the lipid fluidity decreases in the presence of carbamylcholine and (+)-tubocurarine. This is in agreement with the conclusion made above in observing variations in frequency of the lipid vibration at 1084 cm^{-1}.

The uncertainty level of the I_{2880}/I_{2850} lipid ratios

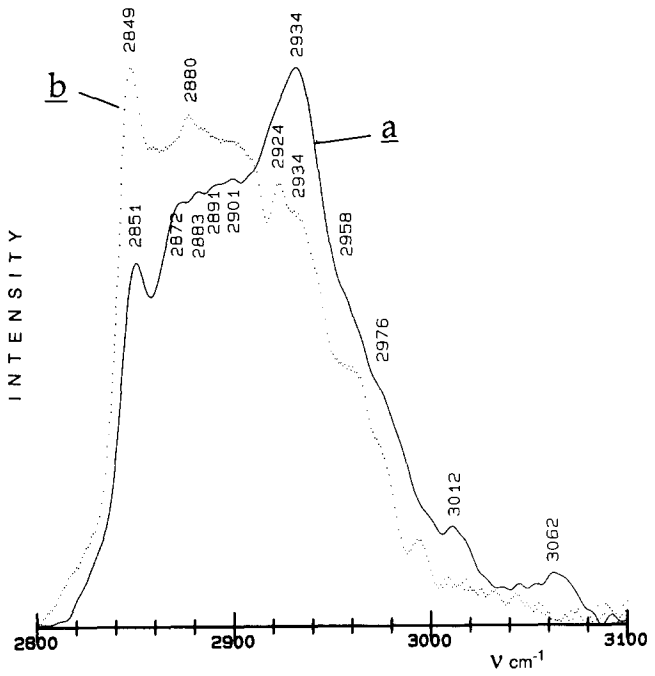

Fig. 7. Raman spectrum of membrane fragments from *T. marmorata* with native AcChR (a) and the spectrum of lipid extract (b).

could be derived from Fig. 6 where the three forms of the AcChR are compared. From this figure it can be seen that, on average, the percentage contributions of the signals in this region do not differ more than 2–3%. The only larger difference we found is about 8% which therefore could be accepted as a significant difference. Thus, a change in surface ratio I_{2880}/I_{2850} exceeding this magnitude may also be accepted as a significant difference. If we compare the values obtained for the three samples, these differences are 16% and 24% for AcChR with carbamylcholine and with (+)-tubocurarine, respectively, compared to the native form. We modified artificially the amplitude of the deconvoluted line at 2880 cm^{-1}. For the normal case this amplitude ratio (I_{2880}/I_{2850}) was 0.947, i.e., 94.7%. We modified it to 64.6%, which is a considerable modification. This alteration caused a surface change from 50.9% to 62.6%, corresponding to an 11% change in the contributions of the vibrations, which should also be regarded as considerable. This value, compared with the 16% minimal change in the case of AcChR-carbamylcholine complex, indicates that the statement about the altered lipid fluidity can be considered as the real one.

Discussion

In this study we analyzed Raman spectra of alkali-treated AcChR-rich membrane fragments from *Torpedo marmorata* electric organ. The secondary structure determination presented here in Table I has been performed with the aim to have a reference allowing comparison between native and carbamylcholine or (+)-tubocurarine-treated membranes. Upon addition of the agonist carbamylcholine or the competitive antagonist (+)-tubocurarine, detectable changes could be observed in the Raman vibrations corresponding to the amide-I region, as well as in aromatic residues, disulfides bridges conformations, methionine residues, (C-C) groups and membrane lipids.

Regarding the differences between AcChR in its native state and in AcChR-carbamylcholine or AcChR-(+)-tubocurarine complexes (Table I), the following could be stated. There is no significant difference in the secondary structure contributions between native AcChR and AcChR-carbamylcholine complex, if one compares the total α-helical and the total β-sheet contents. These observations are in agreement with the notion that the conformational changes accompanying allosteric transitions in regulatory enzymes involve quaternary and tertiary structure but not extensive secondary structure changes [62]. Such quaternary structure [63] and tertiary structure [64] changes have been observed between the resting and desensitized states of *T. marmorata* nicotinic receptor. The data obtained here with carbamylcholine, an agonist stabilizing the desensitized conformation at equilibrium, further suggests that the secondary structure of the receptor is not subject to large modifications. On the contrary, equilibration of AcChR-rich membranes with the competitive antagonist (+)-tubocurarine leads to detectable difference in the amide-I region (Table I) which may be interpreted as reflecting different contributions of α-helical and reversed turns to the secondary structure. These differences (8% and 7%) are only to a small extent greater than the average error of the determination procedure ($\pm$5%). Although the possibility that the secondary structure changes in the presence of (+)-tubocurarine cannot be ruled out, the stability of the β-sheet content (about 25% in all of the three cases), the increased contributions of turns (from 18% to 25%) and that of undefined structures (from 11% to 13%) might indicate some structural changes other than that of the secondary structure. Just recently, during manuscript preparation, we came upon some IR-studies [65] on the AcChR, indicating that there is no alteration of the secondary structure content in the AcChR-(+)-tubocurarine complex although large conformational changes have been detected in tritium-hydrogen exchange measurements [66]. Thus, our finding could also be interpreted as changes in the frequencies of the vibrational characteristics for the different types of structures, which can lead to a slight alteration of the values determined, or which can increase the error limit of the determination.

In addition to the information obtained from the amide-I region, our Raman spectra provided information about the environment of aromatic amino acids,

the (C-C) bonds, the methylene and methyl groups of the aliphatic side chains, as well as the disulfide (S-S) and cysteine (C-S) bonds.

Our spectra indicate that in the membrane fragments with native AcChR the tyrosine residues partially reside on the surface of the molecule 'exposed' to the aqueous medium, although it is not possible to quantify the proportion of 'exposed' and 'buried' residues. In comparison, the Raman spectrum of AcChR-carbamylcholine complex suggests 'exposed' tryptophan residues, while those of the native AcChR and the receptor with (+)-tubocurarine are shown 'buried'. Different exposition and environment of the (C-C) groups of valine and leucine residues are shown by the alteration of frequencies of the 940 and 960 cm^{-1} doublet (Fig. 1).

It was indicated [67] that in the receptor α-subunits three disulfide bridges could be possible: Cys-128 to Cys-142, a sensitive disulfide between Cys-192 and Cys-193 and a disulfide in the lipid bilayer between Cys-412 and Cys-418. Cys-222 is left unpaired and has been associated with a hydrophobic pocket in the receptor which, when alkylated, blocks channel activity. The sensitive disulfide has been associated with receptor activation by agonists. It was demonstrated that the agonists and antagonists binding sites are either identical or both closely situated within 1 nm of this disulfide [68–73].

In the Raman spectrum, three frequencies reflect the alteration in the (S-S) disulfide bridges. A frequency near 510 cm^{-1} corresponds to a *gauche-gauche-gauche* configuration of the -C-C-S-S-C-C- linkage; a *trans-gauche-gauche* configuration gives a frequency near 525 cm^{-1} and a *trans-gauche-trans* is near 540 cm^{-1}. It is not possible to correlate correctly these three vibrations with the established three disulfude bridges mentioned above.

In the Raman spectrum of the membrane fragments with native AcChR (Fig. 1), the vibration at 547 cm^{-1} is the strongest one and indicates a predominant *trans-gauche-trans* configuration. The other two vibrations appear as weak shoulders. In the spectrum of the membrane fragments with AcChR-carbamylcholine complex, the 547 cm^{-1} vibration does not change but the other two frequencies appear to be modified, suggesting some modifications which could probably concern the sensitive disulfides between Cys-192 and Cys-193. Binding of the (+)-tubocurarine to the AcChR changes strikingly the aspect of this region. The three vibrations increase in frequencies and there is a broadening which provides strong evidence for considerable conformational changes in the local geometry of all disulfide bridges. Such line broadening was also observed upon lyophilisation and thermal denaturation of α-lactoglobulin, as well as when lysozyme was denatured by LiBr and SDS [34,37].

The lipid composition of AcChR-rich membranes has been analyzed by Popot et al. [26] who found a rather high cholesterol/phospholipid weight ratio (0.40). The phospholipid composition of the AcChR-rich membranes seems to be comparable with the known phospholipid composition found in other cell membranes. In most of the cases, lipid phase-transition has been obtained in the 0–35°C temperature interval. In spite of the similar lipid composition, no phase transition has been obtained in this temperature interval for AcChR-rich membranes [2]. Two factors could cause this unusual phenomenon, namely the high cholesterol concentration and the very high protein concentration in the AcChR-rich membranes. It was shown that cholesterol added to lipids, broadens the lipid transition [57].

Reconstitution experiments, involving many different lipids in purified acetylcholine receptors from *Torpedo californica* have revealed a striking dependence of the ion channel permeability properties on the lipid composition [74–76]. In general, the receptor requires the presence of negatively-charged lipids (such as phosphatidic acid) and of sterol-like compounds (such as cholesterol) in order to attain the highest level of functional activity as measured by ion-influx assays in the presence and absence of activating ligands. The reconstitution results provide strong evidence that lipids can directly affect the functional properties of the protein. The lipid composition of native receptor-containing membranes is consistent with the requirement of both cholesterol and negatively-charged lipids.

Since changes in the lipid environment have been shown to affect the structure of the protein, it can be expected that changes in protein structure might affect the lipid environment. It is shown here, that the lipids in the membrane fragments with native AcChR protein molecule have their chains in disordered conformation and their lateral packing is reduced. In order to directly examine the effect of some modifications in the receptor protein structure (due to the interaction with an agonist or an antagonist), we analyzed conformationally sensitive lipid vibrations in the Raman spectra of native AcChR and receptor exposed to carbamylcholine and (+)-tubocurarine.

The vibration characteristic for disordered lipid states which appears in the spectrum of the membrane fragments with native AcChR at 1084 cm^{-1} (Fig. 1A) progressively increases in frequency from native AcChR to AcChR-carbamylcholine (Fig. 1B) and to AcChR-(+)-tubocurarine (Fig. 1C) complexes. This has been interpreted as a decrease of lipid fluidity in the presence of carbamylcholine and, especially in the presence of (+)-tubocurarine. In complete agreement with this observation, the ratio of the two fluidity-sensitive lipid vibrations near 2850 and 2880 cm^{-1} increases from 0.53 (for native AcChR) to 0.69 (AcChR with

carbamylcholine) and to 0.77 (AcChR with (+)-tubocurarine). The increasing values of these ratios provide evidence that the lipid fluidity decreases in the presence of the agonist carbamylcholine and, to a higher extent, in the presence of the competitive antagonist (+)-tubocurarine. These observations contrast with the effects described for lipophilic molecules, such as alkanols and volatile anaesthetics, which were found to increase lipid fluidity [77–79] upon interaction with multiple sites located at the boundary between lipids and receptor protein [80,81]. In the present study, we used an agonist (carbamylcholine) and a competitive antagonist ((+)-tubocurarine) known to interact with only two binding sites per receptor molecule which are distinct from the local anesthetic binding sites (reviewed in Refs. 3–6). Although a direct interaction of carbamylcholine and (+)-tubocurarine with lipids cannot be definitely ruled out, the relation between these molecules and lipids appears to be rather indirect and probably results from their preferential binding to a conformation of the receptor molecule which affects membrane fluidity and differs from the conformation of the receptor in native membranes. All of the above arguments support the interpretation that the secondary structure of AcChR does not change due to interaction with carbamylcholine, but a restructuration occurs when AcChR interacts with (+)-tubocurarine. The overall conformation of AcChR-carbamylcholine is closer to the native AcChR than that of AcChR-(+)-tubocurarine. At the same time, there are many well defined alterations in the side chains of proteins and also in the conformation of lipids when either carbamylcholine or (+)-tubocurarine bind to AcChR. They suggest altered protein–lipid interaction as well.

In summary, the reported vibrational Raman spectral data provide evidence for predominant contribution of α-helical secondary structure in the membrane fragments with native AcChR molecule, for a disordered liquid-like state of membrane lipids, and for conformational effects of carbamylcholine and (+)-tubocurarine on AcChR protein and on lipids.

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