Biochem. J. (1992) 288, 421–426 (Printed in Great Britain)

Protein stability and interaction of the nicotinic acetylcholine receptor with cholinergic ligands studied by Fourier-transform infrared spectroscopy

Gregorio FERNANDEZ-BALLESTER,* Jose CASTRESANA,† Jose-Luis R. ARRONDO,† Jose A. FERRAGUT* and Jose M. GONZALEZ-ROS*‡

* Department of Neurochemistry and Institute of Neurosciences, University of Alicante, 03080 Alicante, Spain, and † Department of Biochemistry, Faculty of Sciences, University of the Basque Country, 48080 Bilbao, Spain

Based on the conformational dependence of the amide-I i.r. band, this paper explores the use of Fourier-transform i.r. spectroscopy methods to probe structural features of proteins present in native membranes from *Torpedo* highly enriched in acetylcholine receptor (AcChR). The interference of water absorbance on the amide-I spectral region has been eliminated through isotopic exchange by freeze-drying the membranes in the presence of trehalose to avoid protein denaturation induced by drying, followed by resuspension in deuterated water. AcChR-rich membrane samples prepared in such a way maintained an ability to undergo affinity-state transitions and to promote cation translocation in response to cholinergic agonists, which are functional characteristics of native untreated samples. The temperature-dependence of the i.r. spectrum indicates a massive loss of ordered protein structure, occurring at temperatures similar to those reported for thermal denaturation of the AcChR by differential scanning calorimetry and by thermal inactivation of α-bungarotoxin-binding sites on the AcChR [Artigues, Villar, Ferragut & Gonzalez-Ros (1987) Arch. Biochem. Biophys. **258**, 33–41], thus suggesting that the observed i.r. spectral changes correspond to alterations in the structure of the AcChR protein. Furthermore, the presence of detergents as well as cholinergic agonists and antagonists produces spectral changes that are also consistent with the alterations in AcChR protein structure expected from previous calorimetric studies. In contrast with the information obtained by calorimetry, i.r. spectroscopy allows the contribution of secondary structural changes to be distinguished from the overall change in protein structure. Thus prolonged exposure to cholinergic agonists, which drives the AcChR protein into the desensitized state, produces only negligible alterations in the amide-I band shape, but increases substantially the thermal stability of the protein. This suggests that rearrangements in the tertiary or quaternary structure of the protein are more likely to occur than extensive changes in secondary structure as a consequence of AcChR desensitization.

INTRODUCTION

The nicotinic acetylcholine receptor (AcChR) from *Torpedo* is a transmembrane glycoprotein composed of four different polypeptide subunits (α, β, γ and δ) in a 2:1:1:1 stoichiometry (see refs. [1–4] for review). Binding of cholinergic agonists to the appropriate binding sites on extracellular domains of the AcChR elicits the formation of a transient cation channel, 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.

AcChR channel activation and the corresponding functional responses can be described in great detail through several different techniques (see [5] and references therein). However, information on the structural changes between the different conformational states of the AcChR protein has been more elusive. Prediction methods based on hydropathy analysis of the known protein sequence have been used in model building [3,6–9]. Experimentally, however, only a low-resolution molecular model for the AcChR in the resting and desensitized states has emerged from electron-microscopy studies [10,11]. In the absence of high-resolution data, spectroscopic and calorimetric techniques have provided useful information on certain aspects of AcChR structure. For instance, changes in the fluorescence of intrinsic or extrinsic probes [12–14] or in the transition temperature of protein denaturation [15,16] have suggested that the AcChR in the desensitized state acquires a conformation different from that in the resting state. Such methods, however, can only detect changes in the overall protein arrangement which cannot be ascribed to more specific alterations in secondary, tertiary or quaternary structural features. Use of other techniques such as c.d. or Raman spectroscopy [17–19], which could potentially provide information on the protein secondary structure, has usually been restricted because of excessive scattering or lipid contributions, thus being limited to study of the protein in a detergent-solubilized form or reconstituted into vesicles made of a single lipid component.

In this paper, we have made use of the conformational sensitivity of the protein amide-I i.r.-absorbance band [20,21] to probe structural features of AcChR present in *Torpedo* native membranes. The interference of water i.r. absorbance (1645 cm^{-1}, [22]) on the protein amide-I band has been eliminated by using ^2H_2O instead of H_2O as the solvent. The strong amide-I band, comprising the 1600–1700 cm^{-1} spectral region, results primarily from stretching vibrations of C=O groups in peptide bonds [20], 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 [21]. Thus the amide-I band contours of proteins represent complex composites of spectral components of characteristic frequencies, which have been correlated in H_2O and ^2H_2O with different secondary structures in both soluble and membrane-bound proteins [23–26].

Abbreviations used: AcChR, acetylcholine receptor; αBgt, α-bungarotoxin; PTSA, pyrene-1,3,6,8-tetrasulphonic acid (tetrasodium salt).

‡ To whom correspondence should be addressed.

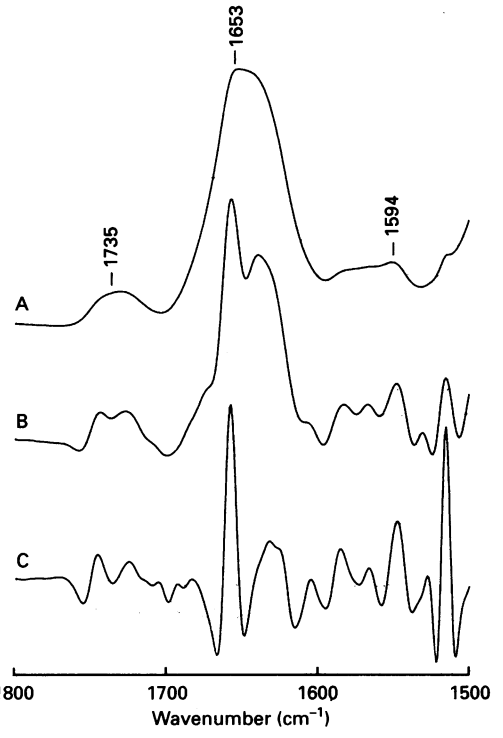

Fig. 1. Representative i.r.-absorbance spectrum of native AcChR-rich membrane samples in ^2H_2O

The original, deconvoluted and derivative spectra are indicated by traces A, B and C respectively. AcChR-rich membranes (approx. 20 mg of protein/ml) in H_2O media (10 mM-Hepes, pH 7.4, 100 mM-$NaNO_3$) were submitted to isotopic exchange by the centrifugation/resuspension procedure, as indicated in the Materials and methods section. The ^2H_2O media used to resuspend 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 spectra of the ^2H_2O media alone (without AcChR-rich membranes) were subtracted from those of the membrane-containing samples and the spectra were recorded at 20 °C, unless stated otherwise.

MATERIALS AND METHODS

Carbamoylcholine chloride, *d*-tubocurarine and deuterium oxide (^2H_2O, 99.9% by atom), were purchased from Sigma. α-[^{125}I]Bungarotoxin (α-Bgt) was from New England Nuclear and α-[^{125}I]Bgt binding to AcChR-rich membranes was measured by using a DEAE-cellulose filter-disc assay [27].

Preparation of AcChR-rich membranes

The electric organ of *Torpedo marmorata* was used to prepare membranes highly enriched in AcChR, as previously described, including alkaline extraction of peripheral membrane proteins [16]. These membranes were suspended in either 10 mM-Hepes buffer, pH 7.4, containing 100 mM-$NaNO_3$ or 10 mM-Tris/HCl, pH 7.4, containing 100 mM-NaCl, and had specific activities of approx. 4 nmol of α-Bgt bound/mg of protein. SDS/PAGE indicated that more than 70% of the protein bands present in these membranes corresponded to the characteristic polypeptide pattern of purified AcChR [16].

I.r. measurements

AcChR-rich membranes for Fourier-transform i.r. spectroscopy were prepared at 20–25 mg of protein/ml, in the buffers described above. Exchange of H_2O with ^2H_2O in the membrane samples was carried out by either (i) submitting the samples to at least two centrifugation/resuspension cycles in ^2H_2O buffers or (ii) freeze-drying the membranes in the presence of trehalose added to avoid protein denaturation induced by drying (routinely at 5 mg of trehalose/mg of membrane phospholipid) and rehydrating in the desired volume of ^2H_2O. Trehalose is a non-reducing sugar present at high concentrations in living animals and plants able to survive complete desiccation, and has been shown to protect soluble [28] as well as membrane [29] proteins against denaturation induced by dehydration.

Fourier-transform i.r. spectroscopic measurements were taken in a Nicolet 520 instrument equipped with a DTGS detector. Samples were placed into a thermostatically controlled demountable cell (Harrick, Ossining, NY, U.S.A.) with Ca_2F windows. The path-length used was 50 μm and the sample chamber was continuously purged with dry air. A minimum of 216 scans per sample was taken, averaged, apodized with a Happ-Genzel function and Fourier-transformed to give a nominal resolution of 2 cm^{-1}. The spectra were then transferred to a personal computer, where standard procedures for Fourier self-deconvolution and Fourier derivation were carried out [30]. Derivation was performed using a power of 3, breakpoint of 0.3. Band-narrowing self-deconvolution was performed by using a Lorenztian bandwidth of 18 cm^{-1} and a resolution enhancement factor of 2.0.

AcChR functional measurements

The ability of the AcChR to undergo agonist–affinity-state transitions was examined (before or after freeze-drying of the membrane samples and in the presence or absence of trehalose) by monitoring the rate of α-Bgt binding to the AcChR-rich membranes [31]. The concentrations of AcChR and α-Bgt in all assays were 0.1 μM (in terms of α-Bgt-binding sites) and 0.25 μM respectively. To induce AcChR desensitization, the membranes were preincubated with 1 μM-carbamoylcholine, for 30 min, before the addition of α-Bgt.

Agonist-mediated cation influx through the AcChR was monitored by using a 'stopped-flow/fluorescence quenching' procedure [32]. Loading of the fluorophore pyrene-1,3,6,8-tetrasulphonic acid (PTSA) (Kodak) into AcChR vesicles was accomplished through a rapid freezing/slow thawing technique and the external fluorophore was removed from the fluorophore-containing vesicles by chromatography on a Sepharose 6MB column. Rapid-kinetics measurements of Tl^+ influx through AcChR membranes loaded with PTSA were obtained as described previously [32], except that a HiTech SF-51 stopped-flow instrument was used, under otherwise identical conditions.

RESULTS AND DISCUSSION

Fig. 1 (spectrum A) shows the 1800–1500 cm^{-1} region of the i.r. spectrum of AcChR-rich membranes in a ^2H_2O medium, in which the ^2H_2O isotopic exchange has been carried out through several centrifugation/resuspension cycles. The 1800–1500 cm^{-1} spectral region exhibits three main absorbance bands that contain information on the structure of membrane components [33]: the band centred at 1735 cm^{-1} corresponds to vibrations from the carbonyl ester groups of membrane phospholipids, and those centred at 1653 cm^1 and 1549 cm^{-1} are the amide-I band and a residual amide-II band respectively, corresponding mostly to peptide-bond vibrations. In aqueous media, the amide-II band arises mainly from in-plane N—H bending vibrations and when protons are exchanged by deuterium, it shifts to around 1457 cm^{-1}, where it overlaps with H—O—^{2}H absorption. Nevertheless, the protein NH groups unable to undergo deuterium exchange still produce a residual amide-II band at 1549 cm^{-1}, the intensity of which is an indication of how difficult the access of the solvent to the protein core is [34]. No time-dependent changes

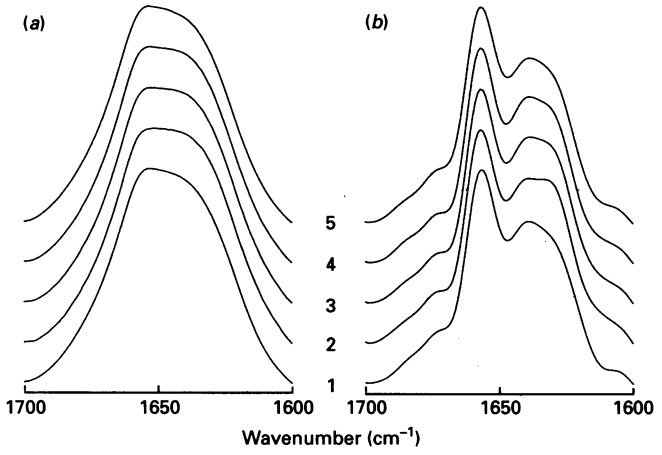

Fig. 2. I.r. amide-I band region of the original (*a*) and deconvoluted (*b*) spectra of AcChR-rich membranes in ^2H_2O

Membranes were prepared by (i) the centrifugation/exchange procedure (trace 1) and (ii) AcChR-rich membranes freeze-dried in the presence of 0, 1, 2 and 5 mg of trehalose/mg of membrane phospholipid, then resuspended in plain ^2H_2O before Fourier-transform i.r. spectroscopic measurements (traces 2–5 respectively)

were observed in the residual amide-II band during the course of the spectroscopic measurements, which indicates that during the time required to prepare the samples (resuspension in ^2H_2O, loading into the cell, purging of the chamber, etc.), the deuterium-exchange process reached an apparent equilibrium.

The information that can be obtained from the amide-I band in the original spectrum is limited by the intrinsic widths of the spectral components contributed by the different protein secondary structures, which are usually larger than their frequency separation and thus produce overlapping. In order to visualize the individual components, narrowing of such bands is accomplished by the application of resolution-enhancement techniques [35–37], such as Fourier self-deconvolution and Fourier derivation (Fig. 1, spectra B and C). On band-narrowing, the amide-I region exhibits maxima at 1605, 1632, 1657, 1680 and 1690 cm^{-1}. Whereas the 1605 cm^{-1} component corresponds to amino acid side-chain vibration [38], all the other maxima have been assigned to vibration of the carbonyl group in peptide bonds within different protein secondary substructures: the 1657 cm^{-1} band is attributed to α-helix, the 1632 cm^{-1} to β-sheet, the 1690 cm^{-1} to turns and the 1680 cm^{-1} includes contributions from turns as well as from the $(0,\pi)$ β-sheet vibration band. A shoulder at 1644 cm^{-1} is also observed, overlapping with the β-sheet peak at 1632 cm^{-1}, which in ^2H_2O has been attributed to random coils. The band assignments made above have been discussed in detail elsewhere for the Ca^{2+}-ATPase in native sarcoplasmic-reticulum membranes [33]. Because native membranes containing a certain amount of non-AcChR proteins (approx. 30%) have been used in this work, the quantitative determination of protein secondary substructures in these complex samples was not pursued. Fig. 1, spectra B and C also shows that the spectral regions corresponding to the phospholipid carbonyl groups and to the residual amide-II band are the result of contributions from several spectral components.

Isotopic exchange by submitting the membrane samples to centrifugation and resuspension in ^2H_2O buffers is, however, a cumbersome procedure since (i) several centrifugations are

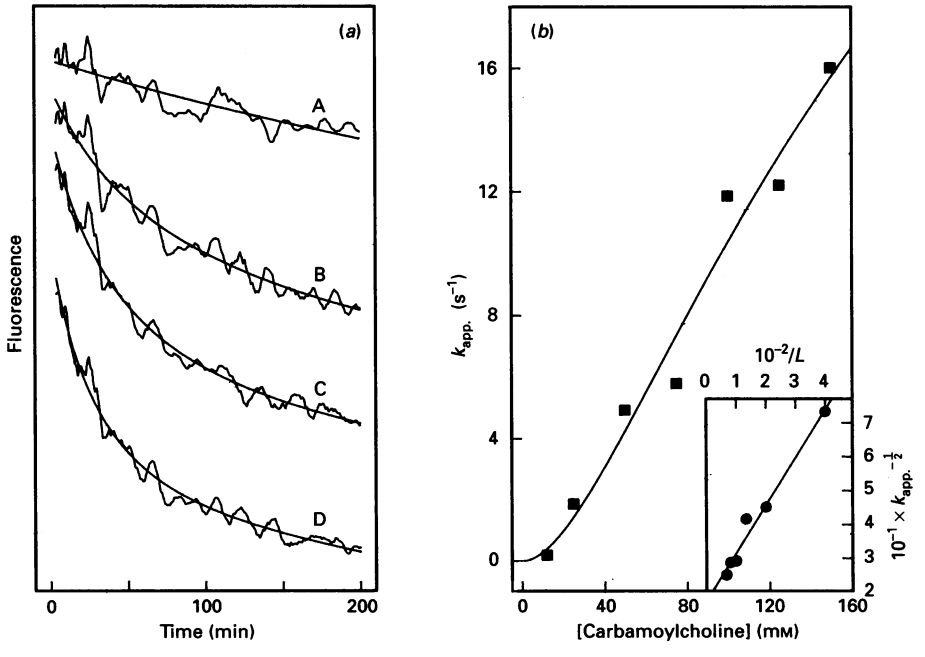

Fig. 3. Agonist-mediated influx of Tl^+ into AcChR-rich membranes containing entrapped PTSA

(*a*) Representative stopped-flow traces corresponding to the Tl^+ influx exhibited by AcChR-rich membranes previously freeze-dried in the presence of 5 mg of trehalose/mg of membrane phospholipid, in response to carbamoylcholine at 0 (A), 50 (B), 100 (C) and 150 μM (D). Preincubation of the membranes with carbamoylcholine to produce AcChR desensitization before the stopped-flow assay completely abolished the influx response. (*b*) Dependence of the apparent rate constant of Tl^+ influx ($k_{app.}$) on the agonist concentration. $k_{app.}$ values were obtained by fitting the experimental traces in (*a*) to the time-dependent Stern–Volmer equation describing the collisional quenching process [32]. The solid line represents the best fit to the equation $k_{app.} = k_{app.}^{max.}[L/(L+K_D)]^h$, where L is the agonist concentration. The Hill coefficient ($h = 1.99 \pm 0.1$) was calculated from the slope of the double log plot of the dependence of $k_{app.}$ versus L. Estimates for the apparent maximum rate constant ($k_{app.}^{max.} = 54.3\ s^{-1}$) and the dissociation constant for the agonist-binding process ($K_D = 1.28 \times 10^{-4}$ M) (inset in *b*) were indistinguishable from those exhibited by membranes that had not been freeze-dried.

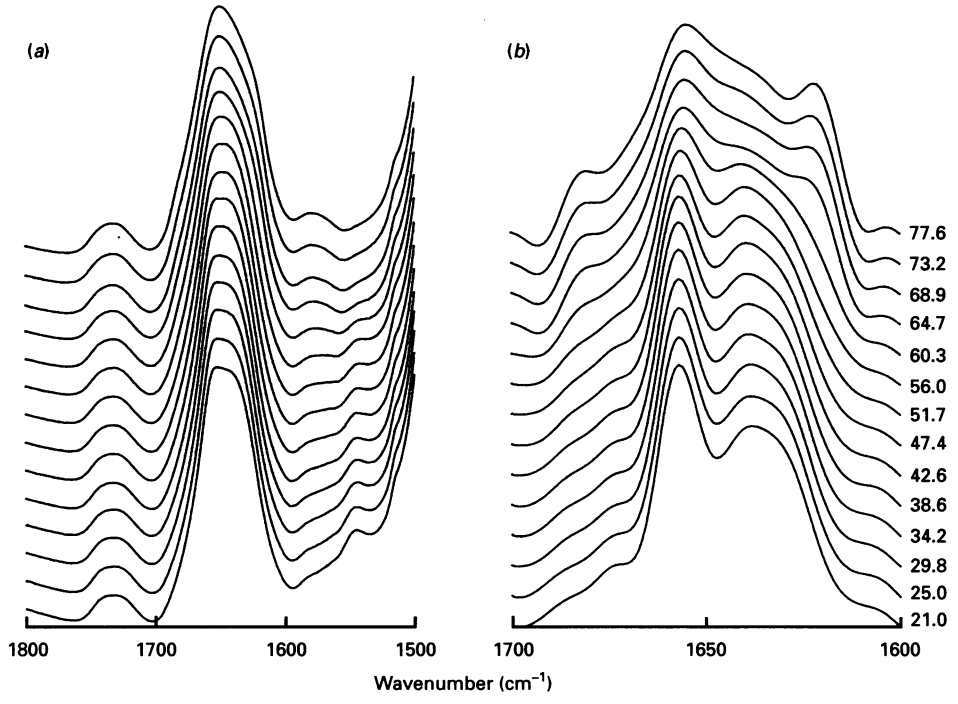

Fig. 4. Temperature-dependence of the i.r. spectrum of AcChR-rich membranes freeze-dried in the presence of 5 mg of trehalose/mg of membrane phospholipid and resuspended in ^2H_2O

(*a*) Original spectra recorded at the indicated temperatures (°C) during a heating cycle. The duration of a heating cycle, including data acquisition and storage at each temperature, was approx. 2.5 h. (*b*) Amide-I band region of the same spectra after Fourier self-deconvolution.

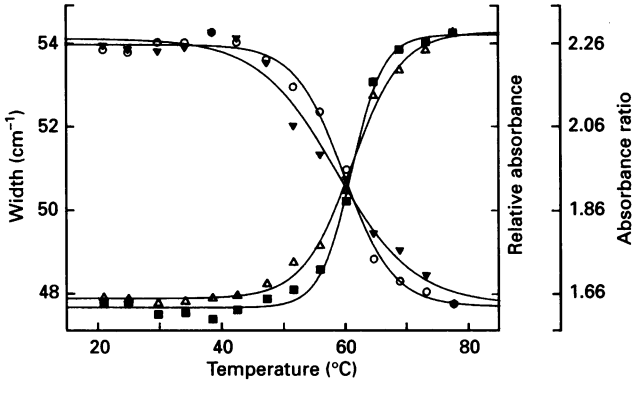

Fig. 5. Monitoring of spectral changes in the amide-I band in the spectra shown in Fig. 4

Changes in the width at half-height (△), in the absorbance at 1620 cm^{-1} (■), in the absorbance at 1657 cm^{-1} (▼) and in the absorbance ratio (1657/1620; ○) show sigmoidal patterns, the inflection points of which converge in a narrow temperature range (59–62 °C).

needed and (ii) membranes or lipid vesicles tend to float in ^2H_2O media and high centrifugal forces have to be used to separate the supernatants. A potential alternative to the ^2H_2O centrifugation exchange is to eliminate the water by freeze-drying of the samples, but this has undesirable structural effects, as evidenced by changes in the amide-I band-shape (Fig. 2) and causes a complete loss of AcChR cation-channel activity. Nevertheless, addition of trehalose to the samples before freeze-drying results in the progressive recovery of the amide-I band shape, which at 5 mg of trehalose/mg of membrane lipid becomes nearly identical with that obtained from samples exchanged by centrifugation (Fig. 2), and preserves the ability of the AcChR to undergo agonist-induced conformational changes (not shown). Moreover, at 5 mg of trehalose/mg of membrane lipids, which has been the trehalose concentration used routinely in most of the experiments, the freeze-dried AcChR sample fully retains the ability to promote cation fluxes on agonist binding, which is characteristic of the native untreated AcChR-rich membranes (Fig. 3). Thus the procedure based on freeze-drying in the presence of trehalose allows for a rapid and convenient preparation of functional AcChR-rich membrane samples suitable for i.r. studies.

To test the ability of our experimental system to detect structural changes in the membrane-bound AcChR protein, i.r. spectra were taken at progressively higher temperatures to induce thermal denaturation of AcChR in these samples (Fig. 4). Thermal denaturation has the advantage that it should imply large rearrangements of the protein structure and that it has been previously described for the AcChR by calorimetric techniques [15,16]. Fig. 4 illustrates changes in the amide-I band that can be observed in either the original (Fig. 4*a*) or the Fourier-deconvoluted (Fig. 4*b*) spectra, indicating a temperature-dependent loss of organized protein secondary substructures, such as the α-helix (1657 cm^{-1}) or β-sheets (1632 cm^{-1}), and the appearance of two components at 1620 and 1684 cm^{-1}, which correspond to interactions between extended chains and have been related to aggregation of thermally unfolded proteins [39,40]. Fig. 4 also shows that the residual amide-II band that remained at 1549 cm^{-1} on ^2H_2O exchange is entirely lost as a consequence of thermal denaturation, thus indicating that the isotopic exchange process can only be completed on protein unfolding. This is probably due to the fact that hydrophobic environments or tightly ordered structures in the native protein, such as the α-helix or β-sheet, partly prevent amide protons from exchanging with ^2H.

The course of the heat-induced events can be monitored at the different wavenumbers corresponding to the appearance or the

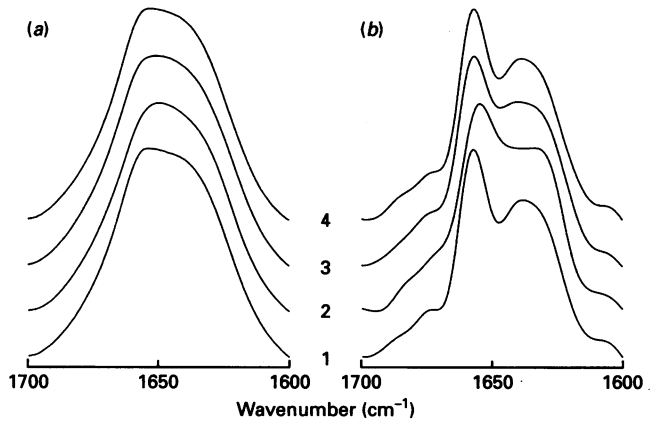

Fig. 6. Effects of detergents on the i.r. amide-I band of AcChR-rich membranes

(*a*) Original spectra; (*b*) Fourier self-deconvolution. All detergents were present at a concentration of 1 % (w/v) which corresponds to detergent to lipid molar ratios of 0.3–0.4. Trace 1 represents a control AcChR-rich membrane sample in the absence of detergent. Traces 2, 3 and 4 correspond to membrane samples in the presence of added SDS, octylglucoside and sodium cholate respectively.

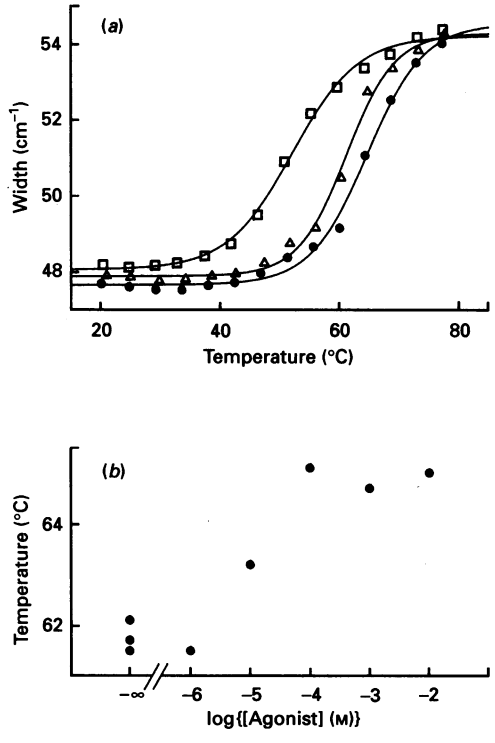

Fig. 7. Effect of a cholinergic agonist (carbamoylcholine) and a membrane detergent (sodium cholate) on the thermal stability of the AcChR in native membranes, monitored by Fourier-transform i.r. spectroscopy

(*a*) Changes in the half-height width of the i.r. amide-I band as a consequence of heating, in the absence of any effector (control; △) and in the presence of cholinergic agonist (1 mM; ●) or sodium cholate at a concentration of 1 % (w/v) (□). (*b*) Concentration-dependence of the stabilizing effect observed with carbamoylcholine. The presence of similar concentrations of the antagonist *d*-tubocurarine produced no effect on the thermal stability of the AcChR-rich membranes (not shown).

disappearance of the protein substructures or by measuring the width of the amide-I band (Fig. 5). In all cases, sigmoidal patterns are observed with inflection points at temperatures within the 59–62 °C range. This is consistent with the AcChR denaturation temperatures observed in aqueous buffers by different calorimetric procedures [15,16], and indicates therefore that the thermal stability of the protein is not greatly perturbed by (i) the use of ^2H_2O media or (ii) the freeze-drying step used in preparing the membrane samples.

Monitoring of the amide-I band by Fourier-transform i.r. spectroscopy was also used in an attempt to detect the presumably smaller protein conformational events expected from the presence of cholinergic ligands or membrane perturbants, including several detergents commonly used in the solubilization of membrane proteins. All samples containing detergents were prepared at a detergent concentration of 1 % (w/v) and therefore the detergent to membrane lipid ratios were within the 0.3–0.4 (w/w) range. Fig. 6 shows that the amide-I band is moderately sensitive to the presence of non-denaturing detergents, such as cholate or octylglucoside, but changes quite dramatically when in the presence of the denaturing detergent SDS. These changes induced by SDS are comparable with those observed for the soluble protein β-galactosidase, where treatment with SDS produces a mixed population of α-helical protein segments in micelles together with aggregated protein [41]. Moreover, Fourier-transform i.r. spectroscopic monitoring of thermal denaturation in membrane samples exposed to the presence of cholate, shown in Fig. 7(*a*), indicates that this detergent induces a loss of protein thermal stability (a decrease of almost 10 °C is observed in the temperature corresponding to the inflection point of the sigmoidal curve shown in Fig. 7*a*), which is similar to the cholate-induced protein-destabilization phenomenon previously observed with calorimetric techniques [16].

The presence of cholinergic agonists (carbamoylcholine) or competitive cholinergic antagonists (*d*-tubocurarine) at concentrations ranging from 1 μM to 1 mM does not induce significant changes in the spectral shape of the amide-I band, indicating the absence of major changes in the protein secondary structure as a consequence of the presence of cholinergic ligands (spectra not shown). This has been confirmed by experiments similar to those described here, but carried out with purified and reconstituted AcChR instead of native AcChR-rich membranes (J. Castresana, G. Fernandez-Ballester, A. M. Fernandez, J. L. Laynez, J. L. R. Arrondo, J. A. Ferragut & J. M. Gonzalez-Ros, unpublished work), which indicate that only cholinergic agonists are able to induce minor reproducible changes in the protein secondary structure. The slightly different observations obtained with the native membranes and the reconstituted AcChR can be accounted for by the presence of non-AcChR proteins in the former, which also contribute to the amide-I band, thus partly obscuring the detection of the minor effects on the AcChR secondary structure caused by the ligands.

Monitoring of thermal denaturation in the presence of cholinergic ligands shows that the agonist carbamoylcholine induces significant thermal stabilization of the protein, which is translated in an increase of up to 3–4 °C in the temperature corresponding to the inflection point of the sigmoidal curve describing the heat-induced loss of protein structure (Fig. 7*a*). Such stabilizing effects of carbamoylcholine are readily detectable at concentration of the ligand higher than 1 μM and become maximal in the 0.1–1 mM range (Fig. 7*b*). Conversely, the presence of similar concentrations of *d*-tubocurarine produces no detectable effect on the estimated temperature of denaturation of the protein, which remains within the range of those exhibited by the control samples. These observations resemble those related to overall structural changes in the AcChR protein, which are detected by calorimetric techniques in the presence of carbamoylcholine or *d*-tubocurarine [16].

The effects of carbamoylcholine on the thermal stability of the

protein, taken together with the lack of large changes in secondary-structural features discussed above, suggest that the conformational events involved in AcChR desensitization induced by cholinergic agonist, but not by cholinergic antagonists, are most related to alteration in tertiary or quaternary structural features of the AcChR protein. Such a conclusion is consistent with previous studies of tritium–hydrogen exchange kinetics [42] and also with the experimental low-resolution model available for the AcChR [10], which predicts that the conformational changes involved in the production of the AcChR desensitized state by cholinergic agonists mainly imply rearrangements in the relative orientation of the subunits within the protein quaternary structure. Moreover, a predictive model of the AcChR protein [6] proposes conformational changes to explain various levels of opening of the ionic channel of the AcChR, which are based on alteration of the tilting of transmembrane α-helices relative to the membrane bilayer and which therefore should not involve extensive alterations in the protein secondary structure. Similar mechanisms based on tilting of protein subunits have also been proposed to explain the opening and closing of other related ligand-activated ion channels [9].

In summary, we have shown that monitoring of the amide-I band in native membranes by Fourier-transform i.r. spectroscopy is a valid and flexible approach to monitoring whether changes in AcChR secondary structure occur in response to different experimental conditions.

This work was partly supported by grants from the DGICYT (PB87-0790 and PB90-0564), the University of the Basque Country (042.310-E033/90) and the European Community (CT91-0666). G.F.-B. and J.C. were recipients of predoctoral fellowships from the 'Generalitat Valenciana' and the Basque Government respectively. We thank Mr Pascual Sempere for providing the live *Torpedo* and the 'Ayuntamiento de Santa Pola' for the use of their aquarium facilities.

REFERENCES

1. Jones, O. T., Earnest, J. P. & McNamee, M. G. (1987) in Biological Membranes (Findlay, J. B. C. & Evans, W. H., eds.), pp. 139–177, IRL Press, Oxford
2. Barrantes, F. J. (1989) Crit. Rev. Biochem. Mol. Biol. **25**, 436–478
3. Stroud, R. M., McCarthy, M. P. & Shuster, M. (1990) Biochemistry **29**, 11009–11023
4. Galzi, J. L., Revah, F., Bessis, A. & Changeux, J. P. (1991) Annu. Rev. Pharmacol. **31**, 37–72
5. Riquelme, G., López, E., Garcia-Segura, L. M., Ferragut, J. A. & Gonzalez-Ros, J. M. (1990) Biochemistry **29**, 11215–11222
6. Guy, H. R. (1984) Biophys. J. **45**, 249–261
7. Finer-Moore, J. & Stroud, R. M. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 155–159
8. Kosower, E. M. (1987) Eur. J. Biochem. **168**, 431–449
9. Unwin, N. (1989) Neuron **3**, 665–676
10. Unwin, N., Toyoshima, C. & Kubalek, E. (1988) J. Cell Biol. **107**, 1123–1138
11. Toyoshima, C. & Unwin, N. (1990) J. Cell. Biol. **111**, 2623–2635
12. Bonner, R., Barrantes, F. J. & Jovin, T. M. (1976) Nature (London) **263**, 429
13. Kaneda, N., Tanaka, F., Kohno, M., Hayashi, K. & Yagi, K. (1982) Arch. Biochem. Biophys. **218**, 376
14. Gonzalez-Ros, J. M., Farach, M. C. & Martinez-Carrion, M. (1983) Biochemistry **22**, 3807–3811
15. Farach, M. C. & Martinez-Carrion, M. (1983) J. Biol. Chem. **258**, 4166–4170
16. Artigues, A., Villar, M. T., Ferragut, J. A. & Gonzalez-Ros, J. M. (1987) Arch. Biochem. Biophys. **258**, 33–41
17. Moore, W. M., Holladay, L. A., Puett, D. & Brady, R. N. (1974) FEBS Lett. **45**, 145–149
18. Yager, P., Chang, E. L., Williams, R. W. & Dalziel, A. W. (1984) Biophys. J. **45**, 26–28
19. Mielke, D. L. & Wallace, B. A. (1988) J. Biol. Chem. **263**, 3177–3182
20. Susi, H. (1969) in Structure and Stability of Biological Macromolecules (Timasheff, S. N. & Fasman, G. D., eds.), pp. 575–663, Marcel Dekker, New York
21. Susi, H., Timasheff, S. N. & Stevens, L. (1967) J. Biol. Chem. **242**, 5460–5466
22. Mendelsohn, R. & Mantsch, H. H. (1986) in Progress in Protein–Lipid Interactions (Watts, A. & DePont, J. H. M., eds.), pp. 103–146, Elsevier Scientific Publishing Co., Amsterdam
23. Surewicz, W. K. & Mantsch, H. H. (1988) Biochim. Biophys. Acta **952**, 115–130
24. Braiman, M. S. & Rothschild, K. J. (1988) Annu. Rev. Biophys. Biophys. Chem. **17**, 541–570
25. Jackson, M., Haris, P. I. & Chapman, D. (1991) Biochemistry **30**, 9681–9686
26. He, W. Z., Newell, W. R., Haris, P. I., Chapman, D. & Barber, J. (1991) Biochemistry **30**, 4552–4559
27. Smidth, J. & Raftery, M. A. (1973) Anal. Biochem. **52**, 349
28. Crowe, J. H., Crowe, L. M., Carpenter, J. F. & Wistrom, C. A. (1987) Biochem. J. **242**, 1–10
29. Crowe, J. H., Crowe, L. M. & Chapman, D. (1984) Science **223**, 701–703
30. Moffatt, D. J., Kaupinnen, J. K., Cameron, D. G., Mantsch, H. H. & Jones, R. N. (1986) Computer Programs for Infrared Spectrophotometry, NRCC Bulletin 18, National Research Council of Canada, Ottawa
31. Gonzalez-Ros, J. M., Sator, V., Calvo-Fernandez, P. & Martinez-Carrion, M. (1979) Biochem. Biophys. Res. Commun. **87**, 214–220
32. Gonzalez-Ros, J. M., Ferragut, J. A. & Martinez-Carrion, M. (1984) Biochem. Biophys. Res. Commun. **120**, 368–375
33. Arrondo, J. L. R., Mantsch, H. H., Mullner, N., Pikula, S. & Martonosi, A. (1987) J. Biol. Chem. **262**, 9037–9043
34. Englander, S. W. & Kallenbach, N. R. (1984) Q. Rev. Biophys. **16**, 521–655
35. Kaupinnen, J. K., Moffatt, D. J., Mantsch, H. H. & Cameron, D. G. (1981) Appl. Spectrosc. **35**, 271–276
36. Mantsch, H. H., Casal, H. L. & Jones, R. N. (1986) in Spectroscopy of Biological Systems (Clark, R. J. H. & Hester, R. E., eds.), pp. 1–46, Wiley and Sons, Chichester
37. Cameron, D. G. & Moffatt, D. J. (1987) Appl. Spectrosc. **41**, 539–544
38. Chirgadze, Y. N., Fedorov, O. V. & Trushina, N. P. (1975) Biopolymers **14**, 679–694
39. Rial, E., Muga, A., Valpuesta, J. M., Arrondo, J. L. R. & Goñi, F. M. (1990) Eur. J. Biochem. **188**, 83–89
40. Surewicz, W. K., Leddy, J. J. & Mantsch, H. H. (1990) Biochemistry **29**, 8106–8111
41. Muga, A., Arrondo, J. L. R., Bellou, T., Sancho, J. & Bernabeu, C. (1992) Arch. Biochem. Biophys., in the press
42. McCarthy, M. P. & Stroud, R. M. (1989) Biochemistry **28**, 40–48

Received 6 March 1992/10 June 1992; accepted 17 June 1992