# Fourier Transform Infrared Difference Spectroscopy of the Nicotinic Acetylcholine Receptor: Evidence for Specific Protein Structural Changes upon Desensitization†

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ABSTRACT: We have previously reported a new method based on Fourier transform infrared spectroscopy for probing conformational changes that occur upon the binding of ligands to the nicotinic acetylcholine receptor (nAChR) [Baenziger, J. E., Miller, K. W., & Rothschild, K. J. (1992) *Biophys. J. 61*, 983–992; Baenziger, J. E., Miller, K. W., McCarthy, M. P., & Rothschild, K. J. (1992) *Biophys. J. 62*, 64–66]. Spectra are recorded using attenuated total reflection both in the presence and absence of agonists. The resulting nAChR "resting-to-desensitized" difference spectra reveal small highly reproducible infrared bands which can arise from vibrations of the agonist and structural changes in the nAChR membrane during the conversion of the receptor from the resting to desensitized state. In this work we have used a combination of different agonists and an antagonist along with isotopic labeling to assign bands in these spectra. nAChR membranes pretreated with the competitive antagonist $\alpha$-bungarotoxin exhibit no bands above the noise level ($\sim 10^{-5}$ au) demonstrating that vibrations of the unbound agonist do not contribute to the normal difference spectrum. In contrast, bands in the resting-to-desensitized difference spectra are identified which can be assigned to the bound agonist, providing a means to probe its interaction and orientation in the binding site. Additional difference bands are due to secondary structural changes of the protein, perturbation of tyrosine(s), and changes in carboxyl groups possibly from Asp and/or Glu residues. Remarkably, some of these spectral changes are similar to those detected during the bleaching of the photoreceptor membrane protein rhodopsin.

The nicotinic acetylcholine receptor (nAChR)[1] from *Torpedo* is the best characterized member of a family of ligand-gated ion channels that performs a central role in signal transduction across postsynaptic membranes. It is a large integral membrane protein of molecular mass ~300 000 daltons and is composed of four homologous subunits in the stoichiometry, $\alpha_2\beta\gamma\delta$. The subunits are arranged pseudo-symmetrically around a central pore that functions as the ion channel, and channel opening requires the binding of two acetylcholine molecules, one to a site on each of the two $\alpha$ subunits [see Stroud et al. (1990) and Galzi et al. (1991a) for recent reviews].

In the absence of ligands, the nAChR exists at equilibrium in two predominant conformations, the resting and desensitized states, which are distinguished pharmacologically by their relative affinities for agonists such as acetylcholine (ACh) and carbamylcholine (Carb). The equilibrium favors the activatable resting state which has a moderate affinity for both ligands, but prolonged exposure to agonists and several noncompetitive antagonists shifts the equilibrium toward the high-affinity nonactivatable or desensitized state (Boyd & Cohen, 1980a,b; Heidmann & Changeux, 1979a,b). Circular dichroism (Mielke & Wallace, 1988) and $^1H/^3H$ exchange experiments (McCarthy & Stroud, 1989a) indicate that the secondary structure and solvent accessibility of hydrogen-bonded secondary structures, respectively, are essentially identical in either conformation. A slight tilting of the $\delta$ subunit toward the $\gamma$ subunit has been detected by cryoelectron microscopy (Unwin et al., 1988), and a change in the susceptibility of specific amino acid residues near the agonist binding site (Galzi et al., 1991b) and in the transmembrane $\alpha$-helices (McCarthy & Stroud, 1989b; White & Cohen, 1989) to photoactivatable probes has been detected upon receptor desensitization. However, a detailed model of the structural changes that lead to the increased ligand affinities has yet to emerge. A major obstacle is the lack of biophysical techniques which can probe conformational changes that occur at the level of single amino acid residues in large (~300 000 daltons) integral membrane proteins.

In this work, we have utilized Fourier transform infrared (FTIR) *difference* spectroscopy, which is a particularly effective method for probing structural differences between a protein's various conformational states (Rothschild et al., 1981; Bagley et al., 1982; Engelhard et al., 1985). The technique has provided detailed information about changes in the structure and protonation state of individual amino acids that occur upon the absorption of light by bacteriorhodopsin, the light-activated proton pump, rhodopsin, the light receptor in vision, and photosynthetic reaction centers [for reviews, see Braiman and Rothschild (1988) and Rothschild (1992)]. Unfortunately, it is not easily applied to proteins whose function is triggered by soluble ligands. Recently, we overcame this limitation and have developed a method for probing conformational changes in the nAChR by combining the attenuated

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[1] Abbreviations: FTIR, Fourier transform infrared; nAChR, nicotinic acetylcholine receptor; Carb, carbamylcholine; ACh, acetylcholine; [$^{18}$O]ACh, $CH_3C^{18}OOCH_2CH_2{}^+N(CH_3)_3$; ATR, attenuated total reflection; $\alpha$-BTX, $\alpha$-bungarotoxin.

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total reflection technique (ATR) with FTIR difference spectroscopy (Baenziger et al., 1992a,b) following earlier work on bacteriorhodopsin (Marrero and Rothschild, 1987a,b). Here, we further demonstrate that the infrared bands observed in the difference of FTIR spectra of reconstituted nAChR films recorded in the presence and absence of cholinergic ligands reflect structural changes that occur in the receptor upon desensitization. Several of the infrared bands are assigned to the vibrations of agonists *bound to the receptor*, while others reflect changes in both secondary structure and in the structure and/or environment of individual amino acid residues.

## MATERIALS AND METHODS

The nAChR was isolated from the electroplaques of *Torpedo californica*, affinity purified to homogeneity, and reconstituted into membranes composed of dioleoylphosphatidylcholine/dioleoylphosphatidic acid/cholesterol in a molar ratio of 3:1:1 (McCarthy & Moore, 1992). The reconstituted membranes (250 $\mu$g of protein) in 5 mM phosphate buffer, pH 7.0, were dried under a stream of nitrogen to form a thin film on the surface of a 50 × 20 × 2 mm germanium internal reflection element (45° aperture angle; Harrick Scientific, Ossining, NY) and equilibrated with buffer (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM $NaH_2PO_4$, pH 7.0). The nAChR membrane films are stable in the presence of aqueous solution and retain the ability to undergo the resting-to-desensitized transition (Baenziger et al., 1992a,b). The nAChR sample was placed in an attenuated total reflection (ATR) sample cell (Harrick Scientific, Ossining, NY) and was maintained at a constant temperature throughout the FTIR experiments by circulating water from an external water bath set at 22.5 °C (NESLAB Instruments, Portsmouth, NH) through an external compartment surrounding the ATR cell. The Carb and ACh are from Sigma (St. Louis, MO) and Aldrich (Milwaukee, WI), respectively. The isotopically labeled acetylcholine was synthesized from $^{18}O$-labeled acetic anhydride ($CH_3C^{18}OOC^{18}OCH_3$) (ICON, Summit, NJ) and dimethylaminoethanol, followed by quaternization with methyl iodide.

FTIR spectra of the nAChR films were recorded on a Nicolet 510P spectrometer (Nicolet Analytical Instruments, Madison, WI) equipped with a MCT detector using attenuated total reflection. Spectra (except where noted) were recorded at 8 $cm^{-1}$ resolution using 1000 scans which took roughly 8 min per spectrum. For the difference measurements, two consecutive spectra of the same nAChR film in the resting state were recorded with buffer *flowing continuously* through the sample compartment of the ATR cell at a rate of ~1.5 mL/min. The flowing solution was then switched to an identical one with the addition of either 50 $\mu$M Carb or 10 $\mu$M ACh. After allowing 2 min for buffer exchange, a spectrum was recorded of the desensitized state. The differences between the two resting state spectra and between the consecutive resting and desensitized state spectra were each calculated and stored, and the flowing solution was switched back to buffer without agonist to wash either the ACh or Carb from the film. After a 23-min washing period, the entire process was repeated, and as many as 20 of the corresponding difference spectra were averaged. Each experiment was repeated a minimum of three times on new films to check for reproducibility of the results.

## RESULTS

FTIR absorbance spectra of a hydrated nAChR film recorded in the presence (desensitized state) and absence (predominantly resting state) of a desensitizing concentration of Carb are virtually superimposable. The two most prominent peaks of each spectrum are centered at 1651 and 1547 $cm^{-1}$ and are due to the amide I and amide II vibrational modes of peptide groups, respectively (Figure 1, top two traces). The broad amide I band is a superposition of several underlying component bands whose frequencies are characteristic of different types of protein secondary structure (Susi & Byler, 1986). The most intense of these underlying components revealed by spectral deconvolution (not shown) occur near 1655 $cm^{-1}$ and 1640 $cm^{-1}$. These two-component bands likely reflect the existence, in both the resting and desensitized states, of $\alpha$-helix and $\beta$-sheet conformations, in agreement with earlier reports based on circular dichroism (Mielke & Wallace, 1988).

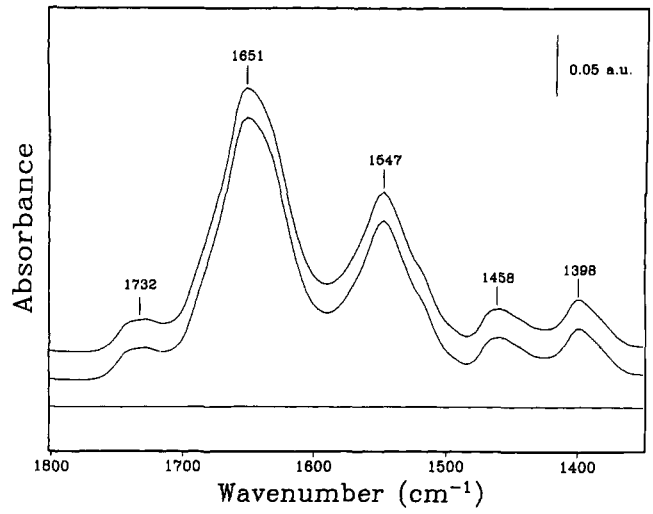


FIGURE 1: FTIR spectra of affinity-purified and reconstituted nAChR membrane films recorded using attenuated total reflection in the presence (middle trace, predominantly desensitized state) and absence (top trace, predominantly resting state) of 50 $\mu$M carbamylcholine. The infrared spectrum of water has been subtracted from both. The bottom trace is the difference of the above two spectra at the same scale.

The difference of the two absorbance spectra recorded in the presence and absence of Carb (referred to as the Carb resting-to-desensitized difference spectrum) exhibits at the scale shown no noticeable features (Figure 1, lower trace) indicating that the conformation of the receptor is very similar in the resting and desensitized states. However, expansion of the absorbance scale 500× reveals many *highly reproducible* positive and negative bands (Figure 2, top trace, bold) which are not present in the difference spectra recorded with only buffer flowing past the film (i.e., no Carb) (Baenziger et al., 1992b). The most intense bands in the Carb resting-to-desensitized difference spectrum have also been observed at a reduced intensity and signal-to-noise ratio in the corresponding difference spectra recorded from native alkaline-extracted membranes from *Torpedo nobiliana* which contain only 15–25% functional nAChR by weight (Baenziger, 1992a). On the basis of parallel fluorescence experiments (Baenziger, 1992b), which demonstrate that similar nAChR films undergo the resting-to-desensitized state transition, it can be concluded that the infrared bands in the difference spectra are associated with nAChR desensitization. Note that positive bands in the difference spectrum could arise from either the vibrations of the desensitized state of the nAChR membrane or the added Carb molecules which can be specifically bound to the receptor, nonspecifically bound to the membrane, or free in solution, while negative bands are due to the infrared absorption of nAChR membranes in the resting state.

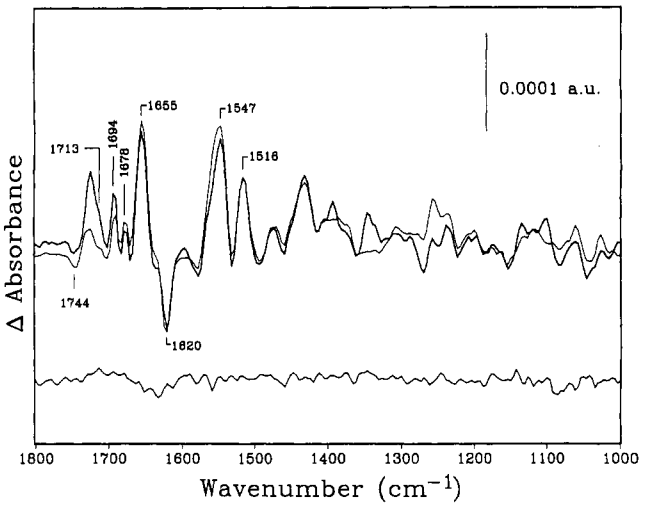


FIGURE 2: (Top) Difference of FTIR spectra recorded from nAChR membrane films in the resting and desensitized states. The "bold" spectrum was calculated from spectra recorded in the presence and absence of 50 $\mu$M carbamylcholine (Carb) and is the average of 37 difference spectra from three separate films. The "fine" spectrum was calculated from spectra recorded in the presence and absence of 10 $\mu$M acetylcholine (ACh) and is the average of 35 differences recorded from two separate films. The spectra were scaled so that the tyrosine band at 1516 cm$^{-1}$ and the Carb and ACh bands near 1475 cm$^{-1}$ have approximately the same intensity. For more details, see Materials and Methods. (Bottom) Difference of FTIR spectra recorded in the presence and absence of 50 $\mu$M Carb from nAChR membranes that were treated with a 10-fold molar excess of $\alpha$-bungarotoxin for 1 h before formation of the membrane film. This spectrum is the average of 20 difference spectra recorded from one film.

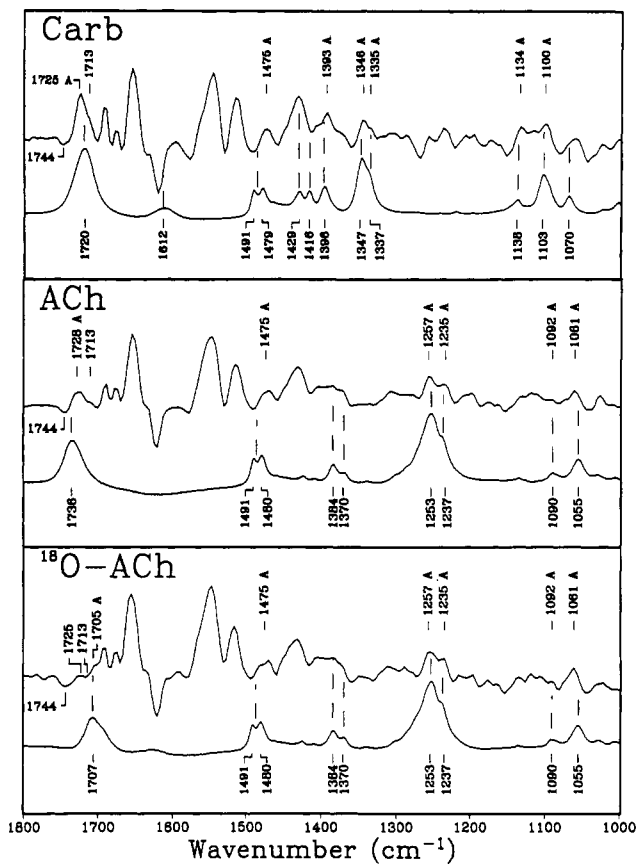


FIGURE 3: Comparison of (top) carbamylcholine (Carb), (middle) acetylcholine (ACh), and (bottom) isotopically labeled acetylcholine ($CH_3C^{18}OOCH_2CH_2^+N(CH_3)_3$); [$^{18}$O]ACh) resting-to-desensitized difference spectra (top traces) with the corresponding solution spectra (bottom traces) of Carb, ACh, and [$^{18}$O]ACh. Each difference spectrum was calculated as described in the legend to Figure 2 except that the [$^{18}$O]ACh difference spectrum represents the average of 30 differences recorded from two films. Bands in the difference spectra assigned to the agonist are indicated by an A. The solution spectra were recorded at 2 cm$^{-1}$ resolution using ATR from 250 mM solutions of Carb and ACh and a 100 mM solution of [$^{18}$O]ACh (pH 7.0). Water bands are subtracted from each solution spectrum. Note that the resting-to-desensitized difference spectra are at 8 cm$^{-1}$ resolution.

In order to test whether unbound or nonspecifically bound Carb contributes to the difference spectrum, we first recorded spectra in the presence and absence of Carb from nAChR membranes *pretreated* with an excess of the competitive antagonist, $\alpha$-bungarotoxin ($\alpha$-BTX) (Figure 2, bottom) (referred to as the $\alpha$-BTX control difference spectrum). $\alpha$-BTX binds essentially irreversibly to each of the two agonist binding sites on the nAChR, competitively inhibits the binding of Carb, and thus prevents the transition from the resting to the desensitized state. Significantly, the essentially flat baseline (noise is less than 0.02 × 10$^{-3}$ au) in the $\alpha$-BTX control difference spectrum indicates that the infrared bands observed in the nAChR resting-to-desensitized difference do not contain contributions from the Carb free in solution in the flowing buffer. In particular, if some spectral differences did arise from the addition of free Carb to the flowing buffer or to Carb's nonspecific membrane interactions, we would expect these contributions also to occur in the $\alpha$-BTX control difference spectrum. The absence of such bands is not surprising since the concentration of Carb in solution was 50 $\mu$M, much less than the minimum concentration of soluble organic molecules detectable by FTIR-ATR using a similar method (C. Cabanting, S. Rankin, N. Gandhi, and K. J. Rothschild, unreported data). Contributions from Carb nonspecifically bound to the nAChR membrane (e.g., Carb which associates with the lipids in the membrane) can also be excluded, assuming that such nonspecific binding is not affected by the presence of $\alpha$-BTX in the binding site. In addition, the essentially flat baseline observed in the $\alpha$-BTX difference spectrum (Figure 2, bottom) demonstrates that there is effectively no loss of membrane from the germanium IRE during the resting-to-desensitization cycle.

We next compared the resting-to-desensitized difference spectra obtained using the agonists acetylcholine (ACh) and carbamylcholine (Carb). Overall, the ACh and Carb resting-to-desensitized difference spectra are similar (Figure 2, top), demonstrating the reproducibility of the data and that the desensitized state induced by each agonist is essentially the same. *However, there are several infrared bands unique to each difference spectrum.* Significantly, almost all of these bands can be assigned to the absorption of the bound ligands (agonists) by comparing the vibrational frequencies of these bands to the frequencies of the corresponding bands in the solution spectra of Carb (Figure 3 top panel, lower trace) and ACh (Figure 3 middle panel, lower trace). For example, intense bands are observed at 1100, 1135, 1346, and 1393 cm$^{-1}$ in both Carb and the Carb-induced difference spectra, while a different set of bands appear near 1055, 1092, 1235, and 1257 cm$^{-1}$ in the ACh and the ACh-induced difference spectrum. *We therefore conclude that we are detecting the vibrations of the bound agonist directly in the resting-to-desensitized difference spectrum.* The assignment of such bands is important not only because it allows one to identify by exclusion bands related to nAChR conformational changes (see below), but also because it provides a direct method to study the structure and orientation of the ligand in the binding pocket similar to studies previously performed on the retinylidene chromophore of bacteriorhodopsin using polarized

infrared spectroscopy (Earnest et al., 1986).

Resting-to-desensitized difference spectra were also measured using isotopically labeled ACh ([$^{18}$O]ACh), $CH_3$-$C^{18}OOCH_2CH_2^+N(CH_3)_3$ (Figure 3, lower panel). This spectrum and comparison with the unlabeled ACh and Carb difference spectra provides a basis for a more complete separation of bands due to the bound agonist and to conformational changes of the nAChR membrane. Preliminary assignment of these bands leads to several interesting conclusions about the nature of the structural changes in nAChR membranes during desensitization.

*Structural Changes in Asp/Glu Groups.* The ester carbonyl stretching vibrations of Carb, ACh, and [$^{18}$O]ACh appear at 1720, 1736, and 1707 $cm^{-1}$, respectively, in the solution spectra of each agonist (Figure 3, lower traces). It can be seen that these bands also contribute positive intensity in the difference spectra (Figure 3, upper traces), but their exact frequency and intensity is masked by overlap with bands due to intrinsic structural changes occurring in the nAChR membrane. For example, in all three cases a negative band at $\sim$1744 $cm^{-1}$ and a positive shoulder near $\sim$1713 $cm^{-1}$ is detected. Overlap with a third positive band near 1725 $cm^{-1}$ would explain why an "apparent" upshift of 5 $cm^{-1}$ occurs in frequency for the Carb ester carbonyl stretch mode (middle panel), whereas a downshift of roughly 5 $cm^{-1}$ occurs for the case of ACh. In the case of [$^{18}$O]ACh which has an ester carbonyl stretch frequency of 1707 $cm^{-1}$, intensity is observed near this frequency in the difference spectrum, whereas a small feature still remains at 1725 $cm^{-1}$. An alternative, but less likely, explanation for these frequency shifts is that a change occurs in the intrinsic frequency of the Carb and ACh ester carbonyl stretch modes reflecting different environments of the ester carbonyl oxygen of Carb and ACh upon binding to the receptor.

The positive and negative bands at 1713, 1725, and 1744 $cm^{-1}$, respectively, are characteristic of the carbonyl stretching modes of protonated carboxylic acids and could reflect a change in the environment and protonation state of aspartic and/or glutamic acid residues. Bands in this region have been found in bacteriorhodopsin (Rothschild et al., 1981), rhodopsin (DeGrip et al., 1988) (see below), and the photosynthetic reaction center (Nabedryk et al., 1990) and in the case of bacteriorhodopsin assigned on the basis of site-directed mutagenesis to specific Asp residues in the protein (Braiman & Rothschild, 1988). Bands observed between 1550 and 1610 $cm^{-1}$ ($CO_2^-$ antisymmetric vibrations) and between 1300 and 1420 $cm^{-1}$ ($CO_2^-$ symmetric vibrations) are also consistent with a change in the protonation state of carboxylic acid residues, but a more detailed analysis is required for a definitive interpretation. Contributions from structural perturbations of lipid ester carbonyl groups should also be considered since these groups absorb in this region. In the case of rhodopsin, this question was examined using reconstitution in ether lipids which do not possess the ester carbonyl group (DeGrip et al., 1988). Similar studies will be necessary in the future for more specific assignment of these bands in the nAChR difference spectrum.

*Secondary Structural Changes.* The frequencies of the two intense positive bands at 1655 and 1547 $cm^{-1}$ (Figure 2) are highly characteristic of $\alpha$-helical conformation (Susi & Byler, 1986, and references therein). The appearance of these bands upon desensitization could reflect an increase in $\alpha$-helical content and/or an alteration in the $\alpha$-helix structure including a change in the net $\alpha$-helix orientation, as suggested by electron diffraction studies (Unwin et al., 1988). The latter effect would produce changes in the intensity of the amide I and II bands since the nAChR membranes have a net orientation parallel to the germanium crystal plane. However, in this case we would expect these bands to have opposite signs, not both positive as observed here (Earnest et al., 1990). An alternative explanation is that the positive amide I and II bands characteristic of $\alpha$-helix are associated with the negative band at 1620 $cm^{-1}$ characteristic of $\beta$-sheet conformation (Susi & Byler, 1988), indicating a transition from $\beta$ to $\alpha$-helical structure. However, two other positive bands characteristic of $\beta$-structure are found at 1694 and 1678 $cm^{-1}$. Note that the overall change in intensity of the amide I and II bands is only $\sim$0.1% of the absolute intensity, indicating a relatively small change in secondary structure involving only a few amino acid residues. On the other hand, if these bands reflect changes in net $\alpha$-helix orientation, the structural change could involve more residues but would be restricted to a very small reorientation.

An additional possibility is that the positive amide I and II bands reflect a small reduction in the overall thickness of the nAChR film thus leading to an increased absorbance upon desensitization. A similar effect is observed in purple membrane films upon changes in pH (Marrero & Rothschild, 1987). However, other bands appearing in the absolute nAChR infrared absorption spectrum, such as at 1732 $cm^{-1}$ (Figure 1), do not appear in the difference spectrum (see Figure 3 lower panel, top trace) as would be expected if a change in film thickness caused the positive amide I and II bands.

*Perturbation of Tyrosine Residues.* The intense positive band at 1516 $cm^{-1}$ is characteristic of an aromatic ring stretching vibration of tyrosine. As discussed below, conserved tyrosine residues are found in the binding site of the nAChR, and it has been postulated that an ionized tyrosine might act as the putative anionic binding site for the quaternary ammonium group of acetylcholine (Pearce & Hawrot, 1990; Pearce et al., 1991). However, a change in the structure of a tyrosinate residue would lead to either positive or negative infrared bands at 1500 $cm^{-1}$ (Chirgadze et al., 1975) which we do not observe. Thus, the intense positive band at 1516 $cm^{-1}$ most likely indicates that there is a strong perturbation or net reorientation of tyrosines upon desensitization. This structural perturbation could reflect a direct interaction between the agonist and tyrosines in the binding site. Further work using polarized infrared spectroscopy should provide a basis for examining the possibility that tyrosines reorient.

## DISCUSSION

In two earlier papers, we demonstrated that FTIR difference spectroscopy can be used to probe conformational changes in the nAChR (Baenziger et al., 1992a,b). A key feature of these experiments is the ability to record spectra using attenuated total reflection (ATR) while flowing buffer past the nAChR membranes with and without the binding ligand which triggers the channel opening and desensitization of the receptor. This approach offers several advantages over other techniques including the ability to measure small quantities of sample, to detect structural changes in individual amino acid residues of a large integral membrane protein the size of the nAChR ($\sim$300 000 daltons), and to obtain information about receptor–drug interactions.

Recently, FTIR difference spectra of nAChR-containing membranes isolated from *T. californica* were obtained using transmittance methods combined with flash-induced release of caged carbamylcholine (Gorne-Tschelnokow et al., 1992). However, strong difference bands from the cage release masked most bands due to conformational changes of the protein,

making it difficult to compare these results to the present work. In addition, the authors report that the caged compounds used caused desensitization of the nAChR prior to agonist release, a problem which is not encountered in the ATR approach.

A first step in analyzing the FTIR difference spectra obtained from nAChR membrane is to separate bands due to binding of the ligand from structural changes triggered by this binding. We have demonstrated here that this is possible by direct comparison with the solution spectra of several agonists and through the use of isotopic labeling of the agonist. A similar approach has also been used to separate protein and retinal chromophore bands in retinal-containing proteins such as bacteriorhodopsin and rhodopsin [see Braiman and Rothschild (1988) and references therein]. In the case of nAChR, however, we also have to include the possibility that bands arise from free agonist in solution and from the nonspecific binding of the agonist (i.e., agonist not in the nAChR-binding pocket). We have examined this possibility by using $\alpha$-bugarotoxin which competitively blocks binding of the agonist to nAChR. The results obtained from these experiments allow us to reach the following conclusions.

*(i) Agonist–Receptor Interactions.* The fact that $\alpha$-BTX abolishes signals in the resting-to-desensitized difference spectrum demonstrates that there are no contributions in the normal difference spectrum from the free agonist in solution which would interfere with the analysis of difference spectra. The absence of such contributions is due to the low agonist concentrations used (i.e., $<50\ \mu M$) and because the thickness of the AChR sample deposited on the Ge ATR crystal is close to the penetration depth of the nonpropagating infrared radiation outside the reflecting crystal. These factors act to minimize contributions from the bulk aqueous solution. Hence, we conclude that bands assigned to the agonist in the resting-to-desensitized difference spectrum arise from the bound agonist. Furthermore, contributions from nonspecific binding of the agonist (i.e., to the membrane lipids) can be excluded since if it did occur such agonists signals would also appear in the control difference spectrum obtained using $\alpha$-BTX. This conclusion is important since it shows that details about receptor–ligand interactions are directly accessible by analysis of the intensity and frequency of agonist bands in the FTIR difference spectra. This includes information about the structure, orientation, and stoichiometry of ligands which interact with the receptor. Additional information can be obtained by using a variety of ligands that are chemically modified or isotopically labeled in these experiments.

*(ii) Structural Changes in nAChR Involve Tyr, Asp, and Glu Residues.* Our results show that FTIR absorbance spectra of nAChR membranes recorded in the presence and absence of a desensitizing concentration of Carb are essentially identical indicating, in agreement with previous studies (Mielke & Wallace, 1988), that the *overall* secondary structure is unchanged upon desensitization. However, at a finer level, once bands due to the bound agonist are taken into account, *FTIR difference spectra* reveal changes in nAChR secondary structure, the environment and/or protonation state of aspartate or glutamate residues, and the structure of tyrosine(s) upon desensitization. These results are of particular interest because conserved aspartic acid and tyrosine residues are found in regions of the amino acid sequence associated with the agonist binding site of the nAChR. These bands might therefore reflect agonist-induced structural changes in binding site residues. It is generally accepted (Michelson & Zeimal, 1973) that the agonist binding domain of the nAChR consists

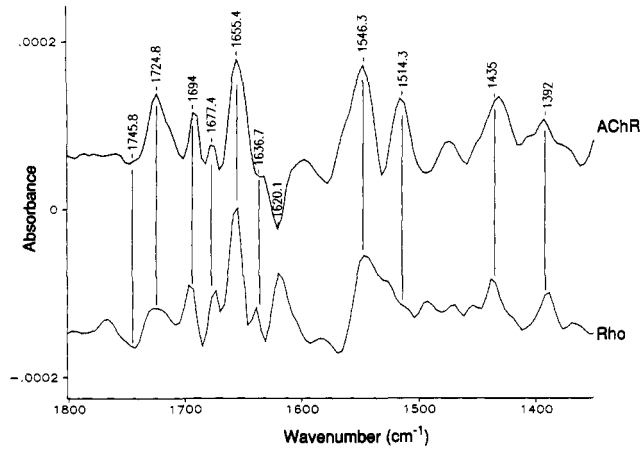


FIGURE 4: Comparison of Carb-induced resting-to-desensitized FTIR difference spectrum (top) (from Figure 3) and the rhodopsin to meta III difference spectrum (bottom) which has been reversed on the *Y*-axis so that negative bands arise from meta III and positive bands from the resting (unbleached) state of rhodopsin. The rhodopsin to meta III difference spectrum was recorded at 8-$cm^{-1}$ resolution on a Nicolet Instruments 60SX spectrometer equipped with an MCT detector as previously reported (Rothschild et al., 1987). The difference spectrum shown is from a 1-min data collection 15 min after bleaching of the sample.

of an esterophilic site which interacts with the ester carbonyl of ACh and an anionic site, possibly an aspartate or tyrosinate (Pearce & Hawrot, 1990; Pearce et al., 1991), which interacts with the cationic quaternary nitrogen. It has also been suggested that the trimethylammonium group of ACh and other agonists bind to a pocket lined with aromatic rings via cation $\pi$-electron interactions (Dougherty & Stauffer, 1990). As discussed by Dougherty and Stauffer, an aromatic ring-lined pocket and cation $\pi$-electron interactions account for much of the data concerning the binding of agonists to the nAChR. This hypothesis is also consistent with the delocalization of the positive quaternary ammonium charge over the three *N*-methyl groups of ACh (Pullman et al., 1971) and for the decreasing cholinergic activity of acetylcholine as the *N*-methyl groups are replaced by *N*-ethyl groups or protons [see Michelson and Zeimal (1973) and references therein]. Furthermore, several aromatic residues are found in the trimethylammonium binding site domain of acetylcholine esterase (Sussman et al., 1991). As discussed, our data are indicative of a structural perturbation of tyrosine and therefore support the concept that one or more of these residues are involved directly in the receptor–agonist interaction.

*(iii) Similarities with Rhodopsin.* A surprising result of this work comes from comparison of the resting-to-desensitized FTIR difference spectrum of AChR with the light-induced difference spectrum of bovine rhodopsin photoreceptor membrane. In the latter case, we can obtain difference spectra at room temperature between rhodopsin before and after exposure to light. Light absorption causes an 11-cis to all-trans isomerization of the chromophore, where, after several rapid steps occurring on the millisecond time scale, the retinal chromophore is still present in the binding pocket (meta II) and subsequently decays after several minutes to a combination of opsin and meta III (Rothschild et al., 1987; DeGrip et al., 1988).

As seen in Figure 4, many spectral regions of the AChR resting-to-desensitized difference spectrum and the reversed rho-to-meta III difference spectrum are similar [note that the *Y*-axis is reversed from the conventional rho-to-meta III difference spectrum so that it represents the difference between the photolyzed rhodopsin (meta III) and the unphotolyzed

rhodopsin (rho)]. Major bands in both spectra are found at frequencies assigned to protonation changes of Asp and Glu groups (1746 and 1725 $cm^{-1}$), the amide I modes (1695, 1677, 1655, and 1637 $cm^{-1}$), amide II mode (1545 $cm^{-1}$), and carboxylate vibrations from Asp/Glu residues (1435 and 1392 $cm^{-1}$) (DeGrip et al., 1988). However, agreement is not perfect, with a strong band near 1620 $cm^{-1}$ appearing with different signs in the two spectra and a strong 1515-$cm^{-1}$ positive band in the AChR difference spectra appearing only weakly in the rho-to-meta III difference spectra. Furthermore, some of these bands may derive partial intensity from agonist or retinal chromophore vibrations (1393 and 1725 $cm^{-1}$ for Carb and 1655 and 1545 $cm^{-1}$ bands from the retinylidene chromophore). Note that similarities were also found between the reversed rho-to-meta II difference spectrum and the AChR resting-to-desensitized spectrum, although the agreement was not as good as for the rho-to-meta III difference spectrum.

An interesting possibility is that similarities in the difference spectra of the two systems reflect some common features in their respective signal transduction mechanisms. Although, rhodopsin and AChR are very different in terms of the currently accepted models for their primary structure and helix packing (i.e., rhodopsin is part of the seven-helix family of receptor proteins, and AChR has four helices for each of its five subunits), there still exist interesting parallels between the two systems.

(a) In both cases, the difference spectra reflect a structural change occurring in a receptor protein between the ligand-free state (i.e., resting state in the case of AChR, opsin, or meta III in the case of rhodopsin where the chromophore is either absent or bound outside the native retinylidene binding site) and the ligand-bound state (i.e., desensitized state of AChR and unphotolyzed state of rhodopsin which contains 11-*cis*-retinal in the binding site).

(b) In both cases, the binding "ligand" (retinal for the unphotolyzed state of rhodopsin and ACh for the desensitized state of AChR) carries a positive charge at one end of the molecule (Schiff base and choline group) and a neutral group at the other end ($\beta$-ionone ring and acetyl group). This fact and other similarities between rhodopsin, the muscaranic acetylcholine receptor, and other G-protein linked receptors which respond to biogenic amines are particularly striking (Oprian, 1992). Interestingly, from this perspective, the unphotolyzed state of rhodopsin is considered "desensitized" whereas the photolyzed state where the retinal has left the binding pocket (meta III and opsin) is "resting".

(c) In both rhodopsin and nAChR there is a transient active state. In nAChR this is triggered by binding of the ligand which causes the channel to open transiently. In the case of rhodopsin, the active state is triggered by light absorption, which causes a series of rapid structural changes in the protein and chromophore that culminates in the transient meta II state which activates the G-protein, transducin.

A simple model which might help account for the spectral similarities between the two systems focuses on the role of the positive charge of the retinal Schiff base and the choline group of ACh in determining protein conformation. This positive charge could form part of a network of hydrogen bonds and ionic interactions which stabilizes one conformational state of a protein, as suggested previously for both rhodopsin (DeGrip et al., 1988) and bacteriorhodopsin (Braiman et al., 1988, Rothschild, 1992). In the case of rhodopsin, the light-activated 11-cis to all-trans isomerization of the chromophore could disrupt these interactions causing the protein to undergo a conformational change and subsequent release of retinal from the binding pocket. In the case of nAChR, the binding of the agonist (which is equivalent to rebinding of 11-*cis*-retinal to opsin) causes a transition to the desensitized state by allowing interactions to be established between the protein and the positively charged choline group.

## CONCLUSIONS

We have further demonstrated the importance of FTIR difference spectroscopy as a probe of membrane protein conformational change. Along with two earlier papers (Baenziger et al., 1992a,b), we have found that extremely small conformational changes induced by the introduction of low concentrations of agonist can be detected in acetylcholine receptor. In contrast to FTIR transmittance measurements on light-triggered membrane proteins such as bacteriorhodopsin, rhodopsin, and photosynthetic reaction center, we have utilized the method of attenuated total reflection which allows soluble ligands to be introduced into a flowing solution which baths the sample. Thus, a wide range of non-light-activated membrane proteins including the family of G-protein receptors such as the $\beta$-adrenergic receptor should be amenable in the future to investigation by FTIR difference spectroscopy. Major conclusions from the present work are as follows:

(i) Both Carb and ACh induce very similar conformational changes in AChR.

(ii) These conformational changes are specific because they are blocked by preincubating the membranes with the irreversible competitive antagonist $\alpha$-bugarotoxin.

(iii) Agonist bands have been assigned in the difference spectrum by using isotope labeling and by comparison with model compound spectra. The bands reflect the specific binding of the agonist to the protein and not its presence in the external medium or nonspecific binding to lipids.

(iv) Several bands which are not affected by isotopic labeling of the agonist or the use of different agonists originate from the conformational changes of AChR. These include bands characteristic of environmental and protonation changes of Asp and Glu carboxyl groups, structural changes in tyrosine groups, and alterations in secondary structure involving $\alpha$-helices.

(v) There are some similarities in the FTIR difference spectra for AChR desensitization and rhodopsin bleaching indicating that common elements may exist in the structural changes which these two proteins undergo despite their widely divergent structure and function.

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