Incorporation of the nicotinic acetylcholine receptor into planar multilamellar films: characterization by fluorescence and Fourier transform infrared difference spectroscopy

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ABSTRACT A method for preparing thin, planar films of nicotinic acetylcholine receptor (nAChR) membranes that retain the ability to undergo the resting to desensitized state transition and that are suitable for spectroscopic studies has been developed. Native, alkaline-extracted nAChR membranes from *Torpedo* are dried under nitrogen on either a plastic microscope coverslip or a germanium internal reflection element (IRE) and then equilibrated with buffer. The drying procedure has no effect on the functional state of the nAChR as judged by a fluorescence assay using the probe ethidium bromide. The times required for an acetylcholine analogue (carbamylcholine), a local anesthetic (dibucaine), and a fluorescent probe (ethidium bromide) to penetrate films of varying degrees of thickness, interact with the receptor, and then to be washed from the films have been established. Under these conditions, the nAChR films can be repetitively cycled between the resting and desensitized states. Both fluorescence and infrared spectroscopy show that the films adhere strongly to either support even with buffer flowing continuously past the film surface. Fourier transform infrared difference spectra calculated from spectra recorded in the presence and absence of carbamylcholine show small, reproducible bands which reflect changes in nAChR structure upon desensitization.

INTRODUCTION

The incorporation of biological membranes into planar multilamellar films provides an opportunity to obtain structural information about membrane lipids and proteins that cannot be obtained from membrane suspensions (Clark et al., 1980; Rothschild et al., 1980). In such films, the lipids and proteins are normally arranged uniaxially around an axis that is perpendicular to the membrane plane and the average orientation of functional groups relative to this axis can be probed using techniques such as x-ray (Gruner et al., 1982), circular dichroism (Muccio and Cassin, 1979; Hsiao and Rothschild, 1980), nuclear magnetic resonance (NMR) spectroscopy (Baenziger et al., 1988), and polarized infrared spectroscopy (Rothschild and Clark, 1979). Biologically active multilamellar films may also be used to investigate membrane protein structure and function by a variety of techniques including Fourier transform infrared (FTIR) difference spectroscopy (Braiman and Rothschild, 1988; Rothschild, 1992).

The aim of this work is to develop a method for producing "biologically active" multilamellar films of nicotinic acetylcholine receptor (nAChR[1]) membranes that are suitable for attenuated total reflection FTIR difference measurements. The nAChR is an integral membrane protein that functions as a cation-selective ion channel in response to the binding of a neurotransmitter, acetylcholine, and can be purified from the electroplaques of *Torpedo* in relatively large quantities. It has been studied by a variety of biophysical, biochemical, and electrophysiological techniques and is the best characterized neurotransmitter receptor/ion channel in terms of both structure and function. Unfortunately, due to the relatively low atomic resolution structural information available for the nAChR (Brisson and Unwin, 1985; Mitra et al., 1989), details of the structural differences between the resting, open, and desensitized states (Unwin et al., 1988; McCarthy and Stroud, 1989; Mielke and Wallace, 1988) and information about the mechanism by which the binding of acetylcholine leads to channel gating and desensitization are limited.

FTIR difference spectroscopy is a particularly effective method for examining the structural differences between a protein's various conformational states (Rothschild et al., 1981; Bagley et al., 1982; Englehard et al., 1985; Rothschild et al., 1983; Mäntele et al., 1985) and could be used to investigate the conformational changes induced in the nAChR by agonists, such as acetylcholine, and by competitive and noncompetitive antagonists, such as *d*-tubocurarine and local anesthetics, respectively. An important requirement of the technique is the ability to repetitively cycle the protein

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[1]*Abbreviations used in this paper:* nAChR, nicotinic acetylcholine receptor; FTIR, Fourier transform infrared; ATR, attenuated total reflection; IRE, internal reflection element; TR, Torpedo Ringer; α-BTX, α-bungarotoxin; Carb, carbamylcholine; Eth, ethidium bromide; Dib, dibucaine; bR, bacteriorhodopsin.

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between different conformations without disturbing the sample inside the FTIR spectrometer. This might be accomplished by forming a thin film of the nAChR membranes on the surface of an internal reflection element (IRE), acquiring spectra using the attenuated total reflection technique (ATR), and then sequentially flowing a buffer either with or without the neurotransmitter acetylcholine (or another agonist or antagonist, et cetera) past the film surface. However, this approach requires a method for fixing the nAChR membranes to the IRE so that they will not dissociate while buffer is flowing past the film. In addition, the fixation method must not affect the structure of the receptor or its ability to undergo conformational changes.

Several methods have been developed for depositing thin, oriented multilamellar films on planar supports including the Langmuir-Blodgett technique (see Kuhn et al., 1972), drying organic or aqueous suspensions with nitrogen or dry air (Fringeli and Günthard, 1976; Rothschild and Clark, 1979), centrifugation (Blasie et al., 1965), and a combination of drying and centrifugation (isopotential spin drying; Clark et al., 1980). Membrane films formed on the surface of a germanium IRE have also been used extensively for studying membrane lipid and protein structure by ATR-FTIR (see Fringeli and Günthard, 1981). Recently, thin membrane films of bacteriorhodopsin were prepared by drying a concentrated solution of purple membrane on a germanium IRE with a stream of dry air (Marrero and Rothschild, 1987*a*, *b*). In the presence of high concentrations of salt (350 mM $CaCl_2$ or 65 mM $LaCl_3$) the films were extremely stable, yet contained functional bR. FTIR difference spectra of the bR films exhibited a high signal-to-noise ratio, even while exchanging the buffer at a rapid flow rate, and provided insight into the effect of pH and ion strength on the structure of bR.

Here, we examine similar methods for the preparation of nAChR membrane films and determine whether these films are suitable for ATR-FTIR difference spectroscopy. Using a fluorescent probe, ethidium bromide (Eth), it is demonstrated that stable films of nAChR membranes that can be repetitively cycled between the resting and desensitized states are formed by drying aqueous suspensions under nitrogen on both plastic coverslips and germanium IRE's and then equilibrating with buffer. Furthermore, the difference of FTIR spectra recorded in the presence and absence of the agonist carbamylcholine (Carb) reveal highly reproducible infrared bands that reflect conformational changes in the nAChR upon desensitization. The results demonstrate that FTIR difference spectroscopy can be used to study conformational changes in proteins whose function is triggered by soluble ligands. A preliminary report of this work has previously been published (Baenziger et al., 1991).

MATERIALS AND METHODS

Sample preparation

The acetylcholine receptor was obtained from freshly dissected electroplaques of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) as described previously (Braswell et al., 1984). Briefly, the tissue was homogenized, centrifuged at 5,500 rpm for 10 min in a Sorvall GSA rotor, the supernatant filtered through cheese cloth and recentrifuged at 10,000 rpm for 90 min. nAChR receptor-rich membranes were prepared by sucrose density gradient centrifugation and were then base extracted (Neubig et al., 1979) to remove peripheral proteins. The nAChR membranes were diluted to a concentration of ~0.5 mg/ml, adjusted to a pH of 11.0 with 0.2 M NaOH, and stirred for 1 h at 4°C. The nAChR membranes were then centrifuged at 35,000 rpm in a Sorvall A-641 rotor and the resulting pellets stored at −80°C until use. The specific activities of the resulting receptor-enriched membranes were determined by the binding of [^{3}H]-ACh (New England Nuclear, Boston, MA) (Braswell et al., 1984), and ranged from 1–2 nmol of ACh binding sites/mg of protein. Protein concentrations were determined by a modified Lowry assay (Hartree, 1972) using bovine serum albumin as a standard. Where indicated, the nAChR membranes were preincubated with a five to ten-fold excess of the competitive inhibitor, α-bungarotoxin (α-BTX) (Sigma Chemical Co., St. Louis, MO).

Planar membrane films were formed by depositing 100 μl of nAChR membranes (1–2.5 mg/ml of protein, see text) in distilled H_2O, pH = 8.0 on plastic microscope coverslips (Bel-Art Products, Pequannock, NJ), or 300 μl of a solution containing ~0.8 mg/ml protein on a 50 × 20 × 2 mm germanium internal reflection element (45° aperture angle; Harrick Scientific Corp., Ossining, NY). The membranes were dried at room temperature for roughly 30 min under dim light with a stream of nitrogen, placed in Torpedo Ringer buffer (TR; 250 mM NaCl, 5mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5mM NaH_2PO_4, pH 7.0) and left to equilibrate at 4°C.

Fluorescence spectroscopy

Steady-state fluorescence spectra were recorded at ambient temperature on an SLM Aminco 48000S spectrometer (SLM Instruments, Inc., Urbana, IL) interfaced to an IBM PS/2 computer. The nAChR films on the surface of a plastic microscope coverslip were aligned vertically in a quartz cuvette containing TR buffer so that the planar surface of the film formed a 45° angle with the excitation light beam. The excitation wavelength was 500 nm with excitation and emission widths yielding a band pass of 8 nm. Emission spectra were ratioed against the emission intensity of rhodamine G. Ethidium bromide, carbamylcholine, and dibucaine (all from Sigma Chemical Co.) were added to the films as 10× stock solutions in TR buffer to final concentrations of 1 μM, 50 μM, and 100 μM, respectively. After the addition of each compound to the cuvette, the buffer was mixed thoroughly using a Pasteur pipette.

Fourier transform infrared (FTIR) spectroscopy

NAChR films, deposited on the surface of a germanium IRE (see above), were fitted in a temperature-controlled ATR cell (Harrick Scientific Corp.). The ATR cell was placed in the sample compartment of a Nicolet 510P spectrometer (Nicolet Analytical Instruments,

Madison, WI) and spectra acquired at a resolution of 8 cm^{-1} using attenuated total reflection. For each difference spectrum, a resting state spectrum was acquired with TR buffer flowing continuously through the sample compartment of the ATR cell at a rate of ~1 ml/min. The flowing solution was switched to 50 μM Carb in TR buffer and after 2 min, a spectrum was recorded of the desensitized state. The film was then exposed to flowing TR buffer for 45 min to remove the Carb and to convert the receptor back to the resting state. The cycle was repeated several times and successive difference spectra averaged. The temperature of the ATR cell was controlled throughout the experiments by circulating water through an external compartment surrounding the cell. The temperature of the circulating water was maintained at 22.5 ± 0.1°C by an external water bath/circulator (NESLAB Instruments, Portsmouth, NH). For a schematic diagram of a similar ATR cell, see Marrero and Rothschild (1987*a*).

RESULTS

Fluorescence assay of the functional state of nAChR suspensions

Fluorescence spectra recorded from solutions of ethidium bromide (Eth) in both the absence and presence of native alkaline-extracted nAChR membrane suspensions are presented in Fig. 1 *a*. Eth has a fluorescence emission maximum at 615 nm (*curve 4*), but upon the addition of the nAChR membranes, the fluorescence intensity increases and the emission maximum shifts to ~605 nm (*curve 2*). Eth binds with low affinity ($K_D \sim 1 \times 10^{-3}$ M) to a well characterized noncompetitive inhibitor site on the resting state of the nAChR, but with high affinity ($K_D \sim 3.6 \times 10^{-7}$) to the same site on the desensitized receptor (Herz et al., 1987; Chinchetru, 1989). Both the increase in fluorescence intensity and the shift in the emission maximum upon the addition of the nAChR membranes are predominantly due to the high-affinity binding of Eth to a small population of desensitized receptors which exists in equilibrium with the resting state nAChR. Eth itself is also a desensitizing agent (K_{des} of 0.72 μM; Herz et al., 1987) and therefore leads to an increase in the relative percentage of the desensitized receptor.

Exposure of the nAChR membranes to the acetylcholine analogue, carbamylcholine (Carb), induces a conformational change in the receptor from a resting to the open state and, within seconds, to the desensitized state. A further increase in the fluorescence intensity of Eth and a shift in its emission maximum to 595 nm is observed (*curve 1*). The Carb-induced response is not observed with membranes that have been preincubated with a five to ten fold excess of the competitive antagonist, α-bungarotoxin (α-BTX; *curve 3*). α-BTX binds essentially irreversibly to the agonist binding site and prevents the agonist-induced desensitization of the nAChR. The changes in Eth fluorescence induced by Carb are therefore due to the desensitization of the

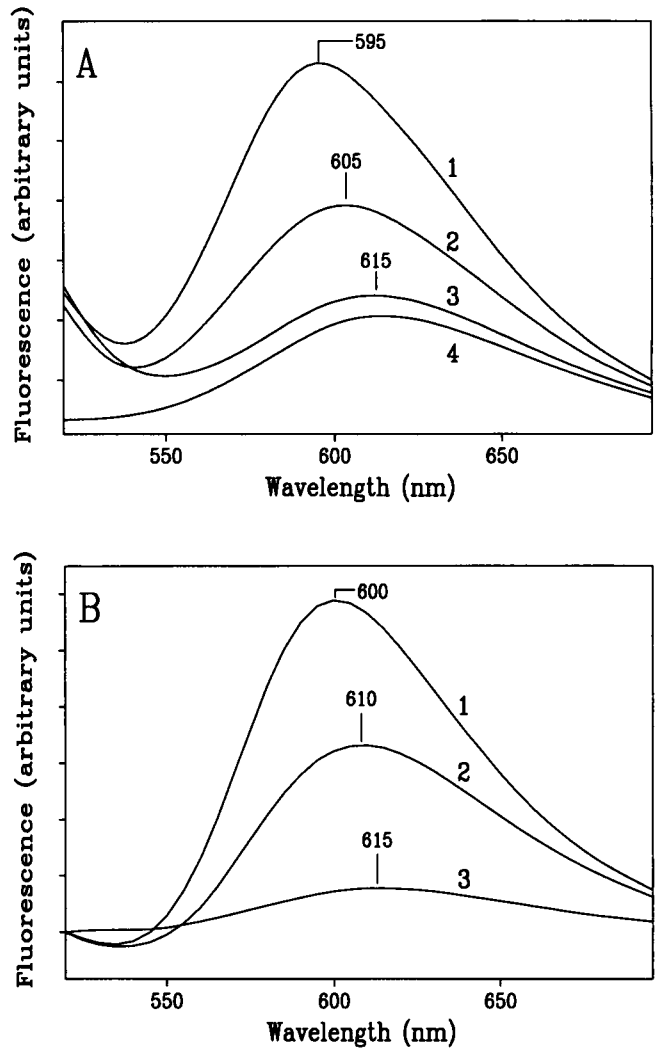

FIGURE 1 Flourescence emission spectra of ethidium bromide (Eth) in TR buffer recorded in the presence of (*a*) nAChR membrane suspensions and (*b*) nAChR membrane films. (*a*) (Curve 1) 0.3 μM native alkaline-extracted nAChR membrane suspensions plus 50 μM carbamylcholine (Carb) and 1 μM Eth; (*Curve 2*) nAChR membrane suspensions plus 1 μM Eth; (*Curve 3*) α-BTX treated nAChR membrane suspensions plus 1 μM Eth and 50 μM Carb; (*Curve 4*) 1 μM Eth. (*b*) Native alkaline-extracted nAChR membranes (0.2 mg protein) were dried under nitrogen on a plastic coverslip and equilibrated with TR buffer. (*Curve 1*) nAChR membrane film plus 50 μM Carb and 1 μM Eth; (*Curve 2*) nAChR film plus 1 μM Eth; (*Curve 3*) α-BTX treated nAChR membrane film plus 50 μM Carb and 1 μM Eth. Note that the raw spectra of the nAChR films were superimposed upon a fluorescence background arising from the scattering of light by the microscope coverslip. The background scattering of the plastic microscope coverslip was subtracted to yield the spectra presented in (*b*). All spectra were smoothed using ten passes of the standard smoothing software (three point, low pass linear digital filter) on the Aminco spectrometer.

nAChR, not a secondary effect, and provide a sensitive, effective method for determining the functional state of nAChR membrane films.

Fluorescence assay of the functional state of nAChR films

Films of native, alkaline-extracted nAChR membranes were initially prepared on plastic microscope coverslips following the same protocol used previously to prepare purple membrane films (see Introduction). The nAChR membranes, suspended in distilled water, were dried under nitrogen and then equilibrating with 350 mM $CaCl_2$. These films were extremely stable, but in the presence of 350 mM $CaCl_2$ showed no Carb-induced increase in the fluorescence of Eth (data not shown). In contrast, membrane films produced by drying membrane suspensions under a stream of nitrogen and then equilibrating with TR buffer still retain the ability to undergo a conformational change. Fluorescence emission spectra of Eth equilibrated with an nAChR film both in the presence and absence of Carb are shown in Fig. 1 *b*. The spectra are similar to those of Eth equilibrated with nAChR membrane suspensions (Fig. 1 *a*). In particular, the film shows a strong increase in the fluorescence emission intensity of Eth and a shift in its emission maximum from 610 to 600 nm upon the addition of Carb (*curve 1* vs *curve 2*), and preincubation with α-BTX abolishes this effect (*curve 3*). The receptor therefore retains the ability to bind the agonist, Carb, to bind the noncompetitive antagonist, Eth, and to undergo the resting to desensitized state transition. Furthermore, the relative intensities of the spectra recorded in the presence and absence of Carb are comparable to the relative intensities of the same spectra recorded for nAChR membrane suspensions indicating that the equilibrium populations of the resting and desensitized states are unperturbed by the drying procedure. Films prepared by drying under nitrogen were stable for days at room temperature and for weeks at 4°C. Drying under nitrogen therefore appears to be an effective method for the preparation of functional nAChR membrane films. Note that we attribute the slight differences in the fluorescence emission maxima observed between the nAChR films and suspensions to errors introduced upon subtracting out the background scattering arising from the microscope coverslip (see Fig. 1).

Cycling between the resting and desensitized states of the nAChR in the membrane films

We examined whether a native, alkaline-extracted nAChR film could be cycled between the resting and desensitized state by monitoring the fluorescence at 595 nm upon the addition and subsequent removal of both Eh and Carb. After the addition of Eth to the film, there is a slow increase in fluorescence emission intensity as the Eth penetrates and binds to the receptor (Fig. 2). In contrast, within 1 min of the addition of Carb, the receptors are completely converted into the desensitized state and a further increase in fluorescence emission intensity is observed. Carb binds with a high affinity to the nAChR ($K_D \sim 1$ μM for the resting state and $K_D \sim 8$ nM for the high-affinity, desensitized state; Herz et al., 1987) and was added at a concentration that is 6,000-fold higher than its K_D for the desensitized state. In contrast, the Eth was added at a concentration roughly equal to its K_D for the desensitized nAChR. As expected, the Carb reaches a concentration that will saturate its binding sites on the nAChR within a shorter period of time.

The nAChR film was then washed extensively over a period of 45 min with TR buffer (no Carb or Eth) to clear both the Eth and Carb from the film and the fluorescence emission intensity returned to its original level. Upon the subsequent addition of Eth there is an increase in fluorescence intensity that follows the same time course and reaches roughly the same intensity as was reached after the addition of Eth in the initial experiment (Fig. 2). *The equilibrium populations of the resting and desensitized receptors are therefore reestablished*

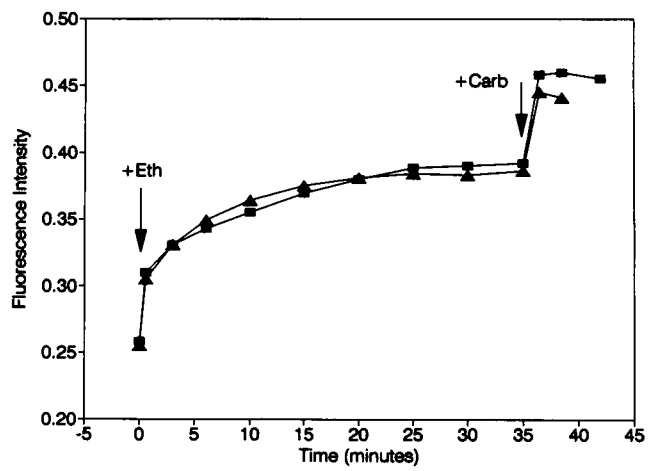

FIGURE 2 Plot of the changes in fluorescence emission intensity at 595 nm with time after the addition of Eth and Carb to a new nAChR membrane film, ■; and to the same nAChR film after cycling the receptor from the desensitized back to the resting state, ▲. The film contained 0.1 mg protein and the Eth and Carb were added to yield final concentrations of 1 μM and 50 μM, respectively. The membranes were recycled from the desensitized to the resting states by exchanging the TR buffer containing both Eth and Carb with TR buffer containing no Eth or Carb. The buffer was exchanged every three minutes for a period of 45 min.

by clearing both the Carb and Eth from the film. Furthermore, the addition of Carb to the recycled film leads to an increase in the fluorescence emission intensity of Eth that is similar to the increase in fluorescence observed upon the initial exposure of the film to Carb. *The "recycled" receptors are still capable of binding Carb and undergoing a conformational change to the desensitized state.* No substantial loss of receptor activity is observed, even after repetitively cycling the receptors between the two conformations over a period of several hours (data not shown) indicating that, by our fluorescence assay, the films remain functional for long periods of time.

The time required to wash the Carb from the film, and thus to convert the receptor back into the resting state, was also investigated by monitoring the fluorescence of ethidium after the buffer containing both Eth and Carb was replaced with a buffer containing only Eth. However, even after numerous washings, the fluorescence emission intensity of the Eth did not decrease (data not shown) suggesting that either Eth stabilizes the receptor in the desensitized state, even after removing the Carb from the bathing solution, or that the Eth is "trapped" in its binding site on the nAChR.

Using an alternative approach, we obtained an estimate of the times required to clear the Carb from films by monitoring the fluorescence of Eth (in the presence of Carb) upon the addition and subsequent removal of a local anesthetic, dibucaine (Dib). Dib has a similar size and structure to that of Carb, and binds competitively with a relatively high affinity (K_D = 1.7 μM) to the same noncompetitive inhibitor site as Eth on the nAChR (Herz et al., 1987). The addition of dibucaine to a nAChR film previously equilibrated with both Eth and Carb leads to a slow decrease in the fluorescence emission intensity of Eth as the Dib penetrates the film and displaces the Eth from its binding site. Exchanging the buffer containing Eth, Carb, and Dib with a buffer containing only Eth and Carb then leads to a slow increase in fluorescence emission intensity back to the same intensity level observed before the addition of dibucaine (not shown). The rate at which the fluorescence intensity was recovered (i.e., the rate for washing the Dib from the film) is dependent upon the film thickness and the frequency with which the buffer in the cuvette was exchanged. With the buffer exchanged every 3 min, films formed using between 0.1 and 0.25 mg of protein had clearance times for the dibucaine that ranged from 30–90 min.

A general estimate of the time required to clear Dib from an nAChR film is roughly 200 min for a film with a surface density of one milligram of protein per cm^2 surface area. The films used for the fluorescence measurements had a density varying from 0.13 to 0.32 mg/cm^2, whereas the films prepared on the germanium IRE's for the FTIR difference measurements (see below) had a surface density of roughly 0.1 to 0.2 mg/cm^2. We found that although the anesthetic is slightly more nonpolar than Carb, the clearance times for dibucaine provide a reasonable approximation of the times required to clear Carb from the nAChR films. Although, more frequent buffer exchange and continuous buffer flow may lead to faster times for recycling the receptor back to the resting state, the results provided the minimum conditions for cycling the receptors during the FTIR difference measurements (see below).

Conformational changes of nAChR membranes detected by FTIR difference spectroscopy

As a prerequisite for FTIR difference measurements, the affinity of the native alkaline-extracted nAChR membrane films for the germanium IRE's was examined using infrared spectroscopy. The infrared spectrum of a dry film of the alkaline-extracted native nAChR membranes is presented in Fig. 3 (*top*) and exhibits several prominent protein bands including the amide I band at 1,655 cm^{-1}, the amide II band at 1,547 cm^{-1}, and the amide A band centered at 3,290 cm^{-1} (not shown). The amide I band frequency at 1,655 cm^{-1}, which is sensitive to protein secondary structure, indicates that there exists a substantial amount of α-helix (Surewicz and Mantsch, 1988). A broad shoulder at 1,640 cm^{-1} and a small shoulder at 1,686 cm^{-1} are also evident, and are characteristic of β structure (Surewicz and Mantsch, 1988). Bands arising from the vibrations of the *Torpedo* lipids include the ester carbonyl C=O double bond stretching mode at 1,738 cm^{-1}, CH_2 bending at 1,462 cm^{-1}, CH_3 antisymmetric bending at 1,443 cm^{-1}, CH_3 symmetric bending at 1,389 cm^{-1}, antisymmetric phosphate double bond stretching at 1,235 cm^{-1}, CH_2 wagging at 1,170 cm^{-1}, C-O stretching due to the fatty acid ester bonds and C-C stretching at 1,165 cm^{-1}, and symmetric phosphate double bond stretching at 1,073 cm^{-1}. Similar bands have previously been identified in transmission infrared spectra of lipids extracted form *Torpedo marmorata* (see Fringeli and Günthard, 1981) and in a spectrum of native nAChR membranes (O'Konski, 1986).

Upon contact with the aqueous solution, an immediate drop in the intensity of both the protein and lipid vibrational bands to ~25% of their intensities in the dry spectrum is observed (data not shown). However, after redrying the film with nitrogen, the intensities of both the protein and lipid bands are recovered, even after the film had soaked for several hours and had been washed extensively with buffer. This phenomenon is most likely due to an increase in the thickness of the film upon

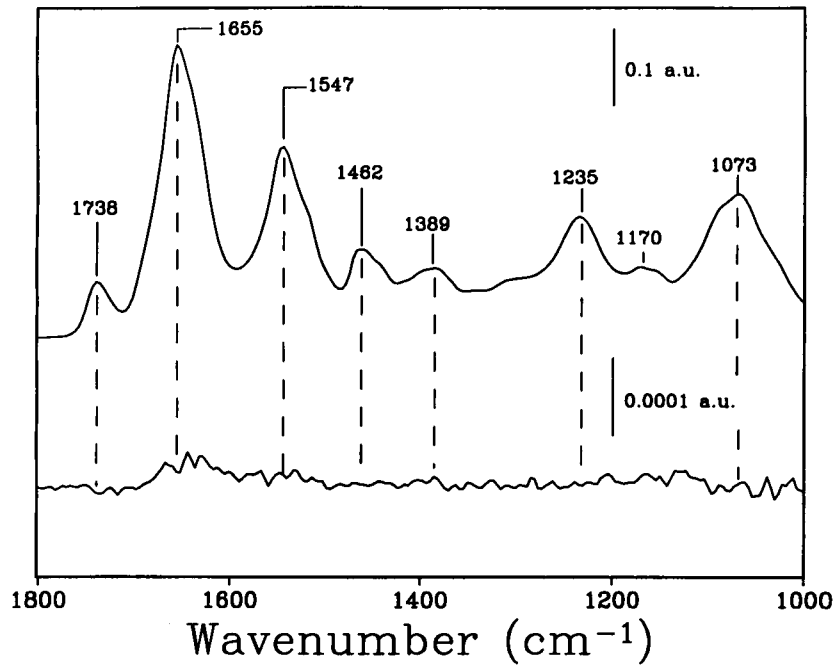

FIGURE 3 (*Top*) FTIR spectrum of a dry film of nAChR membranes recorded at 8 cm^{-1} resolution by the attenuated total reflection technique. (*Bottom*) Infrared difference spectrum of the nAChR membranes calculated from consecutive spectra recorded with buffer flowing through the ATR sample compartment at a flow rate of 1 ml/min. The spectrum is the average of 33 difference spectra, each consisting of 1,000 scans. Note the different absorbance scales.

hydration. The evanescent wave of the infrared beam penetrates only microns into the membrane film thickness, and if the bilayer expands beyond this penetration depth, an apparent loss of intensity will be observed. In support of this interpretation, it was found that films formed using >250 μg of protein did not increase the intensities of the absorbance bands assigned to the nAChR membranes because this primarily increased the thickness of the film beyond the penetration depth of the evanescent wave.

A quantitative measure of the stability of the native alkaline-extracted nAChR membrane films was obtained by recording difference spectra while flowing buffer past the film surface. The difference spectrum presented in Fig. 3 (*bottom*) is the average of 33 difference spectra calculated by subtracting pairs of consecutive 1,000 scan spectra. The difference spectrum exhibits an essentially flat baseline with an excellent noise level ($<5 \times 10^{-5}$ a.u.), although a broad, weak band arising from a positive increase in the intensity of the water band at roughly 1,640 cm^{-1} is present. This band is likely due to small fluctuations in sample temperature. There are no negative bands observed above the noise level that can be attributed to loss of protein or lipid (compare with Fig. 3, *top*) from the germanium surface. At this level of noise (note the difference in the absorbance scales between Fig. 3, *top* and *bottom*), the maximum membrane loss that might occur is <0.05% of the total membrane over a period of 15 min indicating that the nAChR film is extremely stable. It appears that the films formed from native alkaline extracted nAChR membranes are bound essentially irreversibly to the germanium surface.

Difference spectra were also calculated from consecutive spectra recorded before and after the addition of 50 μM Carb to native alkaline-extracted membranes. The level of noise in a single infrared difference spectrum (not shown) is too high to detect bands arising from conformational changes in the nAChR. However, based upon the fluorescence work presented in the previous sections we were able to repetitively cycle the membranes between the resting and desensitized states and signal average consecutive difference spectra recorded before and after the addition of Carb. The resulting signal averaged infrared difference spectrum reveals a number of reproducible bands (Fig. 4, *top*) which are not observed in the difference of spectra recorded with only buffer flowing past the film surface (Fig. 4, *bottom*). Even though the sample contains only 15–25% functional nAChR by weight, it is clear that with signal averaging,

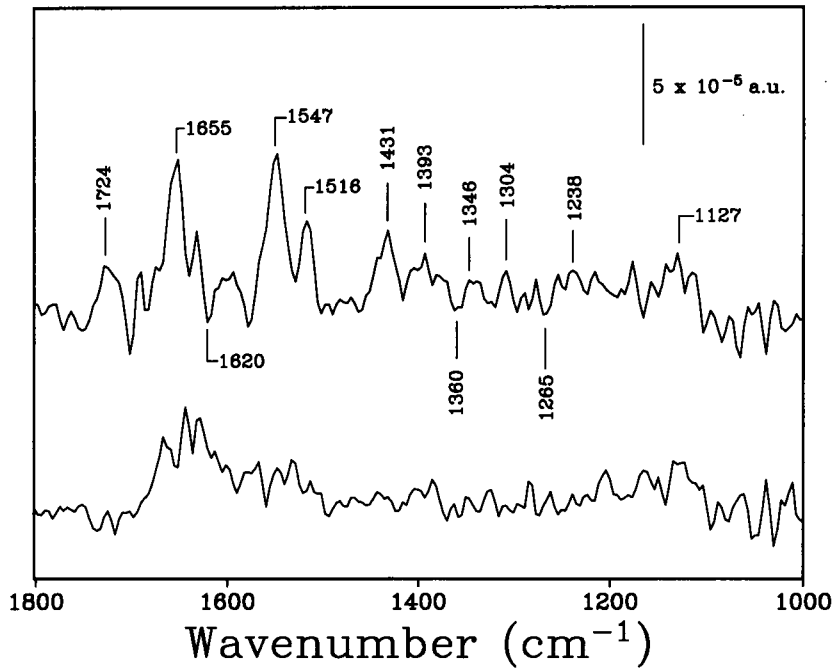

FIGURE 4 (*Top*) Infrared difference spectrum calculated by subtracting a spectrum recorded *before* from one recorded *after* the addition of Carb to a nAChR membranes. The spectrum is the average of 30 difference spectra consisting of 1,000 scans each. For additional details see Materials and Methods. (*Bottom*) same as Fig. 3, *bottom*.

FTIR difference spectroscopy can detect conformational changes in the nAChR membranes upon the addition of Carb. Based upon additional studies (Baenziger et al., 1992), we tentatively assign the bands at 1,655 and 1,547 cm^{-1} to local changes in secondary structure, the band at 1,516 cm^{-1} to changes in the structure and/or environment of one or more tyrosine residues, and the bands at 1,724 and 1,346 cm^{-1} to vibrations of the Carb bound to the receptor. Note that a broad positive band between roughly 1,600 and 1,700 cm^{-1} due to fluctuations in the intensity of the water band is also present (Fig. 4, *top* and *bottom*). The intensity of this water band is sensitive to temperature variations in the sample and leads to a slight variation in the positive and negative intensities of the bands at 1,655 and 1,620 cm^{-1}, respectively.

DISCUSSION

FTIR difference spectroscopy has provided detailed structural information about the conformational changes that occur in light-activated proteins such as bacteriorhodopsin (Braiman et al., 1988; Rothschild et al., 1981; Bagley et al., 1982; Gerwert et al., 1989, and Engelhard et al., 1985) rhodopsin (Rothschild et al., 1983), and the photosynthetic reaction center (Mäntele et al., 1985). These proteins are particularly amenable to analysis using FTIR difference techniques as their conformational changes can be triggered by light without disturbing the sample inside the FTIR spectrometer. In contrast, FTIR difference techniques have not generally been applied to proteins whose function is triggered by soluble ligands. To adapt the technique for probing conformational changes in ligand-activated proteins, a method compatible with FTIR for introducing a soluble ligand, without disturbing the sample inside the spectrometer, is required.

One possible approach for investigating conformational changes in the nAChR is to produce a thin film of the nAChR membranes on the surface of a germanium internal reflection element (IRE) and to acquire spectra using the attenuated total reflection (ATR) technique. ATR spectroscopy allows for the acquisition of infrared spectra in the presence of bulk aqueous solution, and conformational changes can thus be triggered by sequentially flowing a buffer either with or without an agonist past the film surface. This approach requires a method for fixing functional receptors to the germanium IRE so that they will not dissociate while buffer is flowing. However, the development of such a fixation method would not only permit a detailed analysis of conforma-

tional changes in the nAChR, but would also render a variety of membrane receptors amenable to analysis using FTIR difference techniques.

Stability of the nAChR membrane films

Our data clearly indicate that native alkaline-extracted nAChR membranes dried under a stream of nitrogen on either plastic microscope coverslips or germanium IRE's and then equilibrated with TR buffer form thin films that are extremely stable in the presence of aqueous solution. In fact, no loss of membrane from the germanium surface can be detected by FTIR spectroscopy even after flowing buffer past the films for several hours. The membranes are firmly bound to the germanium surface and are thus ideally suited for FTIR difference measurements.

The exact nature of the interactions between the membranes and the germanium surface has not yet been investigated. It was previously found that films of purple membrane require high concentrations of divalent cations for stability (Marrero and Rothschild, 1987*a*), possibly to mask the negative charges of anionic lipids. This is apparently not the case for the native alkaline-extracted nAChR membranes as they are composed predominantly of zwitterionic lipids (Gonzalez-Ros et al., 1982) and would not be expected to exhibit the same forces between the membranes and the germanium IRE's. In contrast to bacteriorhodopsin, the receptor extends substantially beyond the lipid headgroups. Hence, the interactions between the membrane protein and the planar supports may play an important role in determining the stability of the films.

Preliminary FTIR data also show that films of affinity purified nAChR reconstituted into bilayers composed of either dioleoylphosphatidycholine:dioleoylphosphatidic acid:cholesterol, in a molar ratio of 3:1:1, or pure dioleoyl phosphatidylcholine adhere to germanium IRE's with a high affinity (Baenziger et al., 1992). As the native alkaline-extracted and reconstituted membranes differ substantially in their constituent lipid and protein compositions, it can be concluded that the ability to form stable films is not a unique property of the nAChR membranes. Thus, it should be possible to form stable films of membranes containing a variety of biological receptors on germanium IRE's that are suitable for FTIR difference spectroscopy.

Activity of the nAChR films

The fluorescence data indicate that the native alkaline-extracted nAChR membrane films are active. The relative populations of the resting and desensitized states of the nAChR at equilibrium are unaffected by the drying procedure, the receptor retains the ability to bind the agonist carbamylcholine and the noncompetitive antagonists ethidium bromide and dibucaine, and the receptor reversibly undergoes the resting to desensitized conformational change. Furthermore, the difference of FTIR spectra recorded in the presence and absence of Carb reveal highly reproducible infrared bands. Similar bands are not observed in difference spectra recorded with only buffer flowing past the film surface (i.e., no Carb) indicating that they are due to structural changes in the nAChR membranes upon the addition of Carb. FTIR difference spectra obtained from affinity purified and reconstituted nAChR membrane films also reveal similar bands at an enhanced intensity and signal-to-noise ratio providing strong support for this result (Baenziger et al., 1992).

Conformational changes associated with desensitization of the nAChR were previously probed by CD (Mielke and Wallace, 1988), $^1H/^3H$ exchange (McCarthy and Stroud, 1989), and by cryoelectron microscopy (Unwin et al., 1988), but only in the latter study were any changes in structure detected. From the latter study it was suggested that desensitization is associated with a tilting of the transmembrane α-helices relative to the bilayer normal. The positive amide I band at 1,655 cm^{-1} in the infrared difference spectrum could reflect a change in the orientation of transmembrane α-helices. Alternatively, an increase in the α-helical content in the nAChR upon desensitization would also lead to a positive band at 1,655 cm^{-1}. The appearance of a positive band at 1,547 cm^{-1} supports the later interpretation because it would be expected to appear as a negative band if it were associated with an α-helix tilt which gave rise to a positive amide I band (Rothschild and Clark, 1979). The infrared bands observed above the level of noise in other regions of the difference spectrum must reflect either changes in the structure of individual amino acid residues, changes in the structure of the membrane lipids, or the vibrations of the agonist Carb. In particular, the band at 1,516 cm^{-1} is characteristic of tyrosine vibrations, and could reflect the structural changes in tyrosine residues that occur upon ligand binding. It has previously been suggested that tyrosine plays an important role in the agonist binding site of the nAChR (Tomaselli et al., 1991; O'Leary and White, 1991). Bands at 1,724 and 1,346 cm^{-1} correspond to the most intense infrared bands observed in the solution spectrum of Carb. These bands could reflect the vibrations of Carb that is bound to the receptor.

CONCLUSIONS

We have examined the feasibility of forming thin films of nAChR membranes on a germanium internal reflection element (IRE) that are suitable for an attenuated total reflection FTIR spectroscopic study of the structural differences between the resting and desensitized states of the nAChR. Our data show that the membranes form stable films on either plastic microscope coverslips or germanium crystals, and that the drying procedure has no detectable effect on the ability of the nAChR to bind agonists or antagonists, or to undergo the resting to desensitized state transition. Attenuated total reflection difference measurements recorded with the receptor in the resting and desensitized states reveal infrared bands that likely reflect the structural changes in the nAChR that occur upon desensitization as well as the infrared vibrations of the Carb bound to the receptor. A more detailed interpretation of the resting-to-desensitized infrared difference spectrum will require a thorough analysis using films formed from affinity purified and reconstituted nAChR membranes as these films give rise to difference spectra with a substantially improved signal to noise ratio (Baenziger et al., 1992). However, the FTIR difference spectra presented here provide strong evidence that FTIR difference spectroscopy is sufficiently sensitive to detect conformational changes in large (> 250,000 D) integral membrane proteins such as the nAChR, even though the membrane films contain only 15–25% functional nAChR by weight. Our work indicates that FTIR difference spectroscopy can be used to study receptors that are activated by soluble ligands and should therefore prove a valuable tool for investigating conformational changes in the nAChR and other membrane receptors.

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