Probing conformational changes in the nicotinic acetylcholine receptor by Fourier transform infrared difference spectroscopy

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ABSTRACT We have developed a Fourier transform infrared (FTIR) difference method for probing conformational changes that occur upon the binding of ligands to the nicotinic acetylcholine receptor (nAChR). Our approach is to deposit reconstituted nAChR membranes in a thin film on the surface of a germanium internal reflection element, acquire FTIR spectra in the presence of bulk aqueous solution using attenuated total reflection, and then trigger conformational changes by sequentially flowing a buffer either with or without an agonist past the film surface. Using the fluorescent probe, ethidium bromide, it is demonstrated that the method of nAChR film deposition does not affect the ability of the receptor to undergo the resting-to-desensitized state transition. The difference of FTIR spectra of nAChR films recorded in the presence and absence of agonists reveal highly reproducible infrared bands that are not observed in the difference of spectra recorded with only buffer flowing past the film surface . Some of the bands are assigned to changes in protein secondary structure and to changes in the structure of individual amino acid residues. Bands arising from the vibrations of the agonist bound to the receptor are also observed. The results demonstrate that FTIR difference spectroscopy can detect structural changes in the nAChR that occur upon the binding of ligands. The technique will be an effective method for investigating nAChR structure and function as well as receptor–drug interactions.

INTRODUCTION

Fourier transform infrared (FTIR) *difference* spectroscopy can provide detailed information about the structural changes that occur in specific amino acid residues during protein function (1) and has been used extensively to examine changes in the protonation state of amino acid residues during proton transport by the light-activated proton pump, bacteriorhodopsin (2). To apply the difference technique to the nicotinic acetylcholine receptor (nAChR), our approach was to "fix" the nAChR membranes in a thin film on the surface of an internal reflection element (IRE) and to acquire FTIR spectra using attenuated total reflection (ATR). ATR allows for the acquisition of infrared spectra in the presence of bulk aqueous solution and with the membranes fixed to the surface of the IRE, conformational changes can be triggered by flowing a buffer either with or without an agonist past the film surface. We show here using fluorescence spectroscopy that nAChR membrane films formed on plastic microscope coverslips retain the ability to undergo the resting-to-desensitized state transition. We also demonstrate that the difference of FTIR spectra of nAChR films acquired in the presence and absence of the agonist carbamylcholine (Carb) exhibit positive and negative bands that reflect structural changes that occur upon receptor desensitization.

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MATERIALS AND METHODS

The nAChR from the electric organ of *Torpedo californica* was affinity purified and reconstituted into synthetic lipid membranes composed of dioloeoylphosphatidylcholine:dioleoylphosphatidic acid:cholesterol in a molar ratio of 3:1:1 according to the procedure of Ellena et al. (3). Planar membrane films were formed by drying aqueous solutions of the nAChR membranes under nitrogen on either plastic microscope coverslips or germanium IRE's and then equilibrating with buffer. The films formed on plastic coverslips were examined by fluorescence spectroscopy to determine whether drying affects the ability of the receptor to undergo the resting-to-desensitized state transition. FTIR spectra of the nAChR films were recorded using attenuated total reflection in the presence and absence of 50 μM Carb on a Nicolet 510P spectrometer (Nicolet Analytical Instruments, Madison, WI). Details of the experiments are described elsewhere (4).

RESULTS AND DISCUSSIONS

Biological activity of reconstituted nAChR membrane films

The fluorescence emission properties of ethidium bromide (Eth) are sensitive to the conformational state of the nAChR. Eth binds with a low affinity to a noncompetitive antagonist site ($K_D \sim 1$ mM) on the resting state of

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the nAChR, but with a relatively high affinity (K_D = 0.36 μM) to the same site on the desensitized receptor (5). Upon receptor binding, there is a shift in its fluorescence emission maximum from roughly 615 nm to 595 nm with a concomitant increase in the fluorescence emission intensity (4, 5).

Fluorescence emission spectra of Eth in solution and of Eth equilibrated with a reconstituted film are presented in Fig. 1 (curves 1 and 2, respectively). Note that the emission spectra are superimposed on an intense background arising from the scattering of light by the plastic microscope coverslip. The solution spectrum of Eth exhibits the expected emission maximum at 615 nm, but upon equilibration with the nAChR film the intensity of the fluorescence emission increases and the emission maximum shifts to 600 nm (Fig. 1, curve 2). The changes in fluorescence upon equilibration with the film are primarily due to the binding of Eth to a population of receptors that exist in the desensitizated state at equilibrium. The subsequent addition of Carb leads to a further increase in the emission intensity and a further shift in the emission maximum to 595 nm (Fig. 1, curve 3) as Eth binds to receptors that have been converted from the resting to the high-affinity desensitized state. The specificity of the interactions between Eth and the nAChR is confirmed by the subsequent addition of the local anaesthetic, dibucaine, which binds with a relatively high affinity (K_D = 1.7 μM) to the same noncompetitive inhibitor site as Eth on the nAChR (11, 12). Dibucaine displaces the Eth from its binding site and leads to a dramatic decrease in the emission intensity and a shift in the emission maximum to 615 nm (Fig. 1, curve 4). The films formed by drying the reconstituted nAChR membranes under a stream of nitrogen and then equilibrating with buffer therefore contain functional receptors which retain the ability to bind Carb, Eth, and dibucaine, and are capable of undergoing the resting to desensitized state transition.

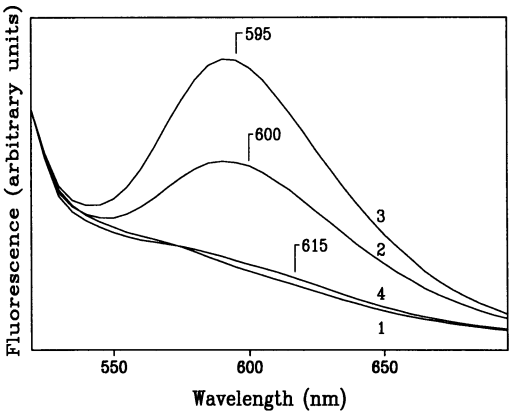

FIGURE 1 Fluorescence emission spectra of ethidium bromide (Eth) in buffer and equilibrated with an nAChR membrane film. (*Curve 1*) 1 μM Eth; (*Curve 2*) 1 μM Eth equilibrated with the nAChR film; (*Curve 3*) 1 μM Eth and 50 μM Carb equilibrated with the nAChR film; and (*Curve 4*) 1 μM Eth, 50 μM Carb, and 100 μM dibucaine equilibrated with the nAChR film. Excitation wavelength is 500 nm.

FTIR difference spectroscopy

The difference of FTIR spectra of affinity purified and reconstituted nAChR membranes recorded in the presence and absence of a desensitizing concentration of the agonist carbamylcholine (50 μM Carb) is presented in Fig. 2, top (referred to as the Carb difference spectrum). The Carb difference spectrum consists of several highly reproducible positive and negative infrared bands which are not present in the difference of spectra acquired with only buffer (i.e., no Carb) flowing past the film (Fig. 2, bottom). Based upon the fluorescence experiments which demonstrate that the nAChR membrane films formed on plastic coverslips undergo the resting-to-desensitized state transition, the infrared bands in the Carb difference spectrum can be assigned to structural changes in the nAChR membranes that occur upon the desensitization of the nAChR. Positive bands represent formation of the desensitized state, including the vibrational bands

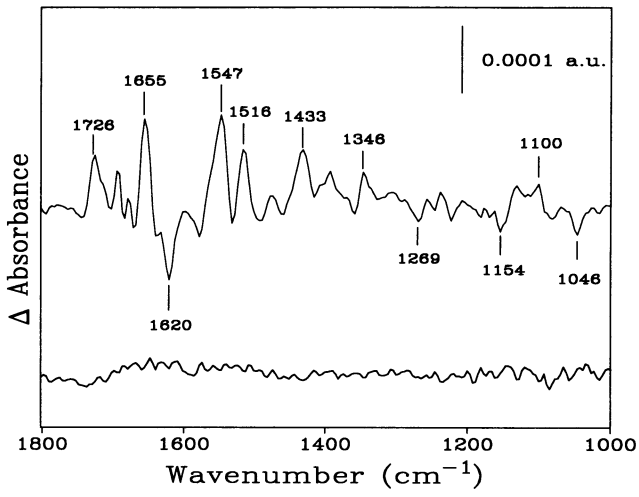

FIGURE 2 FTIR difference spectra of affinity purified and reconstituted nAChR membranes calculated from Top, spectra recorded in the presence and absence of 50 μM carbamylcholine, and Bottom, consecutive spectra recorded with buffer flowing past the nAChR film. Both difference spectra are the average of 20 1,000-scan difference spectra, each recorded at 22.5°C and at a resolution of 8 cm^{-1}.

of Carb bound to the nAChR, and negative bands reflect loss of receptor from the resting state.

Several of the infrared bands are characteristic of protein vibrations. The relatively intense amide I and II bands in the 1,520–1,700 cm^{-1} reflect a change in the secondary structure of the nAChR upon desensitization. The intense band in the 1,700–1,750 cm^{-1} region is due to the ester carbonyl stretching vibration of Carb bound to the receptor whereas other less intense bands could reflect a change in the environment or structure of the ester carboxylic acid of aspartic or glutamic acid residues and/or of lipid ester carbonyls. The band at 1,516 cm^{-1} is characteristic of a tyrosine ring stretching vibration. Conserved tyrosine residues are found within a region of the primary amino acid sequence that forms the ligand binding site and perform a critical role in ligand binding (6–8). The band at 1,516 cm^{-1} may reflect the structural changes that occur in tyrosine residues upon the binding of agonists to the nAChR.

The results clearly demonstrate that FTIR difference spectroscopy can detect structural changes that occur upon the binding of ligands to the nAChR. A detailed analysis of these and other difference spectra recorded from site-specific mutants of the nAChR should provide insight into the nature of the structural changes that occur in specific amino acid residues upon nAChR desensitization and should lead to a more detailed understanding of nAChR structure and function.

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