

A Conformational Intermediate between the Resting and Desensitized States of the Nicotinic Acetylcholine Receptor*

Received for publication, August 4, 2000, and in revised form, November 13, 2000
Published, JBC Papers in Press, November 16, 2000, DOI 10.1074/jbc.M007063200

Stephen E. Ryan‡, Michael P. Blanton§, and John E. Baenziger¶

From the ‡Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada and the §Departments of Anesthesiology and Pharmacology, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

The structural changes induced in the nicotinic acetylcholine receptor by two noncompetitive channel blockers, proadifen and phencyclidine, have been studied by infrared difference spectroscopy and using the conformationally sensitive photoreactive noncompetitive antagonist 3-(trifluoromethyl)-3-*m*-([¹²⁵I]iodophenyl)diazirine. Simultaneous binding of proadifen to both the ion channel pore and neurotransmitter sites leads to the loss of positive markers near 1663, 1655, 1547, 1430, and 1059 cm⁻¹ in carbamylcholine difference spectra, suggesting the stabilization of a desensitized conformation. In contrast, only the positive markers near 1663 and 1059 cm⁻¹ are maximally affected by the binding of either blocker to the ion channel pore suggesting that the conformationally sensitive residues vibrating at these two frequencies are stabilized in a desensitized-like conformation, whereas those vibrating near 1655 and 1430 cm⁻¹ remain in a resting-like state. The vibrations at 1547 cm⁻¹ are coupled to those at both 1663 and 1655 cm⁻¹ and thus exhibit an intermediate pattern of band intensity change. The formation of a structural intermediate between the resting and desensitized states in the presence of phencyclidine is further supported by the pattern of 3-(trifluoromethyl)-3-*m*-([¹²⁵I]iodophenyl)diazirine photoincorporation. In the presence of phencyclidine, the subunit labeling pattern is distinct from that observed in either the resting or desensitized conformations; specifically, there is a concentration-dependent increase in the extent of photoincorporation into the δ -subunit. Our data show that domains of the nicotinic acetylcholine receptor interconvert between the resting and desensitized states independently of each other and suggest a revised model of channel blocker action that involves both low and high affinity agonist binding conformational intermediates.

The magnitude of the cation flux response elicited by acetylcholine at the postsynaptic membrane is dependent upon a variety of factors. These factors include both the number of nicotinic acetylcholine receptors (nAChRs)¹ present in the

postsynaptic membrane and the proportion of these receptors that exist in active *versus* inactive conformations. For example, the nAChR in native *Torpedo* membranes exists in at least two distinct conformations: a low affinity closed (resting) and a high affinity inactive (desensitized) state. In the absence of acetylcholine, the equilibrium between these two strongly favors the resting state with only ~20% adopting the desensitized conformation (1–3). Prolonged exposure to acetylcholine, however, shifts the equilibrium in favor of the desensitized conformation, thus diminishing the postsynaptic response. The magnitude of the cation flux can be modulated further by endogenous factors including receptor phosphorylation (4, 5) and membrane lipid composition (6), which influence the proportion of nAChRs in the resting *versus* desensitized state and/or the kinetics of the resting-to-desensitized conformational transition.

The flux response elicited by acetylcholine is also affected by a class of structurally diverse exogenous compounds collectively referred to as noncompetitive blockers (NCBs). NCBs of the nAChR include both general and local anesthetics, the hallucinogenic drug phencyclidine hydrochloride (PCP), and the frog toxin histrionicotoxin (7, 8). These compounds sterically inhibit cation flux through the nAChR. In some cases, they also modulate the affinity of the nAChR for acetylcholine. PCP and the local anesthetics dibucaine, prilocaine, lidocaine, and proadifen increase the affinity of the nAChR for acetylcholine and are thought to stabilize the desensitized state (9, 10). Other local anesthetics such as tetracaine and adiphenine decrease the affinity of the nAChR for acetylcholine and are thought to shift the equilibrium in favor of the resting state (11). Most NCBs also bind with comparable affinity to the neurotransmitter site as well as to numerous low affinity sites on the periphery of the nAChR (9). The conformational effects, if any, that result from binding to the latter are not well characterized.

We previously examined the structural consequences of local anesthetic binding to the nAChR using FTIR difference spectroscopy (12). The difference between spectra of the nAChR recorded in the presence and absence of the agonist carbamylcholine (referred to as a Carb difference spectrum) exhibits a complex pattern of positive and negative bands that provides a spectral map of the structural changes that occur upon Carb binding and desensitization (12–14, 25). Carb difference spectra recorded in the presence of the desensitizing local anesthetics dibucaine, prilocaine, and lidocaine all exhibit a pattern of band intensity changes that is consistent with the formation of a desensitized nAChR. The spectral changes, however, indicate

* This work was supported in part by grants from the Canadian Institutes of Health Research (to J. E. B.) and by National Institutes of Health NINDS Grant NS35786 (to M. P. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, Microbiology, and Immunology, University of Ottawa, 451 Smyth Rd., Ottawa, ON K1H 8M5, Canada. Tel.: 613-562-5800 (ext. 8222); Fax: 613-562-5440; E-mail: jebaenz@uottawa.ca.

¹ The abbreviations used are: nAChR, nicotinic acetylcholine recep-

tor; Carb, carbamylcholine; FTIR, Fourier transform infrared; PCP, phencyclidine hydrochloride; [¹²⁵I]TID, 3-(trifluoromethyl)-3-*m*-([¹²⁵I]iodophenyl)diazirine; NCB, noncompetitive blocker; MOPS, 4-morpholinopropanesulfonic acid; ATR, attenuated total reflection.

that local anesthetic-induced desensitization occurs upon binding to the neurotransmitter as opposed to the NCB site. The data also suggest that NCB-site binding may lead to the formation of a conformation that is a structural intermediate between the resting and desensitized states, as opposed to the normally assumed desensitized state. Unfortunately, the overlapping binding affinities of the studied local anesthetics for the NCB and neurotransmitter binding sites prevented an unequivocal assessment of the structural changes elicited upon binding specifically to the ion channel pore.

The potential existence of a conformation that is a structural intermediate between the resting and desensitized states has important implications for our understanding of both nAChR conformational equilibria and the mechanisms of agonist-induced conformational change. An intermediate conformation *in vivo* could also play a role in the modulation of a postsynaptic response. To determine unequivocally whether or not such a conformational intermediate does exist, we probe here using both FTIR difference spectroscopy and the conformationally sensitive chemical probe, [¹²⁵I]TID, the structural changes induced in the nAChR by two NCBs, proadifen and PCP, that have distinct affinities for the NCB and neurotransmitter binding sites. Our data show conclusively that the binding of either proadifen or PCP to the NCB site leads to the formation of a conformation that is distinct from either the resting or the desensitized states of the nAChR. In contrast, binding to the neurotransmitter site leads to full desensitization. A revised model of nAChR conformational equilibria is presented.

EXPERIMENTAL PROCEDURES

Sample Preparation—nAChR-rich membranes were prepared by sucrose density centrifugation as described by Chiara and Cohen (15). Affinity purification and reconstitution of the nAChR into membranes composed of 3:1:1 egg phosphatidylcholine:dioleoylphosphatidic acid:cholesterol was as described by McCarthy and Moore (16). The *Torpedo* electric organ was purchased from either Aquatic Research Consultants (San Pedro, CA) or Marinus, Inc. (Long Beach, CA). Egg phosphatidylcholine and dioleoylphosphatidic acid were both from Avanti Polar Lipids (Alabaster, AL). Cholesterol, Carb, and dibucaine were from Sigma. PCP was either a gift from Health Canada or was purchased from Sigma/RBI.

FTIR Difference Spectroscopy—FTIR spectra were recorded using the ATR technique on an FTS-40 spectrometer equipped with a DTGS detector. Each spectrum was recorded at 8 cm⁻¹ resolution using 512 scans, which took roughly 7 min per spectrum. All difference spectra were base-line-corrected between 1800 and 1000 cm⁻¹ and were interpolated to an effective resolution of 4 cm⁻¹. The presented spectra are averages of between 30 and 70 difference spectra recorded, for each experiment, from at least two new films prepared from each affinity purification/reconstitution.

Carb difference spectra were recorded as described in detail elsewhere (12). Briefly, two consecutive resting state spectra of an nAChR film deposited on the surface of a germanium internal reflection element were recorded in the absence of Carb with buffer (250 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 3 mM CaCl₂, and 10 mM Tris, pH 7.0) flowing continuously through the sample compartment of the ATR cell at a rate of ~1.5 ml/min (see schematic in Ref. 12). The flowing solution was switched to an identical one containing 50 μM Carb and after 1 min a spectrum recorded of the desensitized state. The differences between both the two resting state spectra (absence of Carb; control spectra) and the consecutive resting and desensitized (presence of Carb) state spectra were calculated, stored, and the flowing buffer switched back to buffer without Carb. After a 20-min washing period to remove Carb from the film and convert the nAChR back into the resting conformation, the process was repeated many times.

Both proadifen- and PCP-induced structural changes in the nAChR were monitored by recording Carb difference spectra while maintaining the nAChR in continuous contact with either proadifen or PCP (Fig. 1, *bottom schematic*). The structural changes that result from PCP binding to the NCB site were also monitored by recording spectra of the nAChR in the presence and absence of PCP, referred to as PCP difference spectra. The PCP difference spectra were recorded as described above for the Carb difference spectra, except that PCP was used instead

of Carb to induce the conformational change (Fig. 1, *middle schematic*).

The measurement of difference band intensity changes for plotting dose-response curves is difficult because of base-line distortions that can occur in the 1500–1800 cm⁻¹ region as a result of nAChR film instability and temperature fluctuations. Distortions are particularly evident in difference spectra recorded at high concentrations of proadifen, because partitioning of large amounts of this NCB into the bilayer leads to nAChR film instability. Extensive studies have shown that the difference intensity near 1700, 1680, 1637, and 1530 cm⁻¹ is at or near base line, as is evident in the difference spectra recorded at low concentrations of proadifen (Fig. 2, *arrows in the second trace from bottom*). In contrast, higher concentrations of proadifen lead to base-line distortions and an apparent increase in intensity at each of these frequencies (Fig. 3, *arrows in the second trace from bottom*). Peak heights were measured relative to the intensity at an adjacent frequency that is close to the base line and that, based on experience, is not affected by NCB binding to the nAChR. The intensity centered at 1655 cm⁻¹ was measured relative to 1637 cm⁻¹ and is referred to as the 1655/1637 cm⁻¹ ratio. The changes in positive intensity at 1663 cm⁻¹ were best quantified by measuring the changes in negative intensity near 1668 cm⁻¹ relative to 1680 cm⁻¹ and is referred to as the 1668/1680 cm⁻¹ ratio. The binding of proadifen to the neurotransmitter sites was quantified by integrating the intensity of the negative proadifen band centered near 1740 cm⁻¹.

[¹²⁵I]TID Labeling of the nAChR—For photolabeling experiments, *Torpedo* nAChR-rich membranes (1 mg/ml in Vesicle Dialysis Buffer (10 mM MOPS, 100 mM NaCl, 0.1 mM EDTA, and 0.02% NaN₃, pH 7.5) were incubated for 2 h at room temperature with [¹²⁵I]TID (0.4 μM) in the absence or presence of 400 μM Carb, or PCP at various concentrations. In some experiments nAChR-rich membranes were pre-equilibrated with 10 μM α-bungarotoxin for 20 min prior to the addition of PCP, and in some experiments affinity-purified nAChRs reconstituted into asolectin-lipid vesicles were substituted for nAChR-rich membranes. Incubations were performed in 10 × 75-mm glass culture tubes in the dark. The samples were then irradiated with a 365-nm UV lamp (Spectroline EN-280L) for 7 min at a distance of <1 cm, the membrane suspensions transferred to 1.5-ml microfuge tubes and centrifuged at 39,000 × g for 1 h. Pellets were solubilized in electrophoresis sample buffer and nAChR subunits resolved by SDS-PAGE (26) using 1.0-mm-thick separating gels comprising 8% polyacrylamide, 0.33% bisacrylamide. Following electrophoresis, gels were stained with Coomassie Blue R-250, dried, and autoradiographs prepared using Kodak X-Omat LS film at -80 °C in the presence of an intensifying screen (15-h exposure). [¹²⁵I]TID incorporation into AChR subunits was quantified by excising the bands from the dried gel and determining the amount of ¹²⁵I by counting in a Packard Cobra II Gamma counter. The concentration-response data were curve-fitted by nonlinear least-squares analysis using the graphical curve-fitting program Prism (Graphpad Software, Inc.).

RESULTS

FTIR Difference Spectra—The difference between infrared spectra of the nAChR recorded in the presence and absence of the agonist Carb exhibits a complex pattern of positive and negative bands that provides a vibrational map of the Carb-induced structural change (Fig. 1, *top trace*) (13, 25). These difference bands reflect specifically the vibrations of nAChR-bound Carb, as well as vibrational changes associated with both the formation of physical interactions between Carb and the nAChR and the Carb-induced resting-to-desensitized conformational transition (Fig. 1, *top schematic*) (6, 12, 24, 25).

A vibrational map of NCB-induced structural change could similarly be obtained by calculating the difference between spectra of the nAChR recorded in the presence and absence of a NCB (referred to as a NCB difference spectrum; Fig. 1, *middle schematic*). Unfortunately, the bands of interest in NCB difference spectra are usually masked by overlapping bands due to the partitioning of the NCB into the lipid bilayer and a consequent expansion of the nAChR film on the germanium optical element, as shown for the NCB dibucaine in Fig. 1 (*middle trace*; note that PCP is an exception, see below). An alternative approach is to record Carb difference spectra while maintaining the nAChR in continuous contact with the NCB of interest, as shown for dibucaine (Fig. 1, *lowest schematic* and

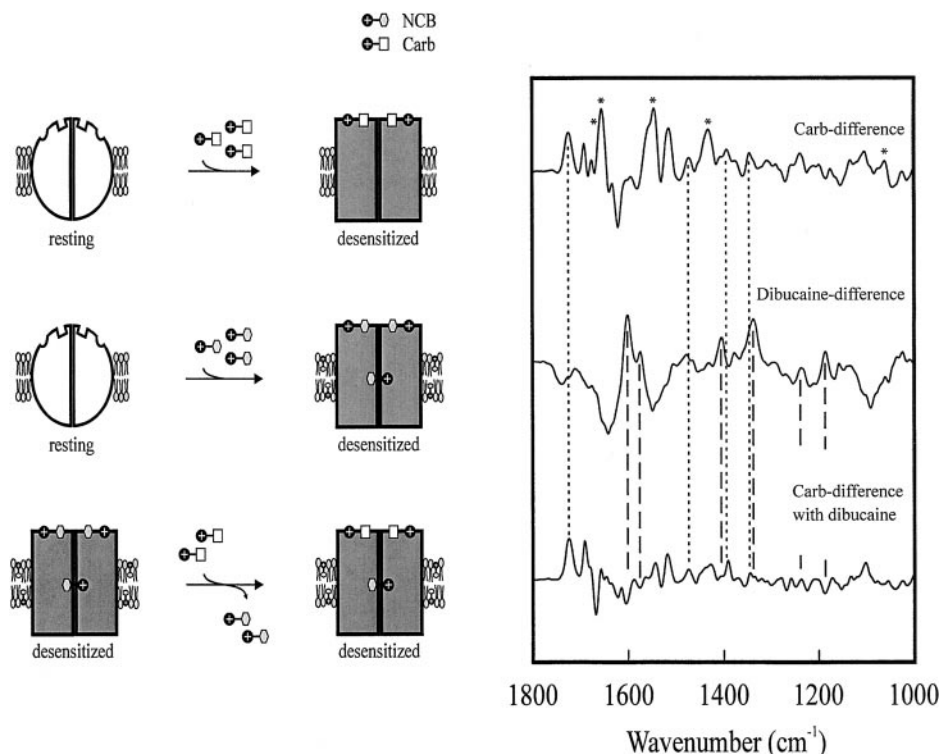


FIG. 1. Conformational changes probed by difference spectroscopy. The difference between FTIR spectra of the nAChR recorded in the presence and absence of Carb (*top panel on left and top spectrum on right*) exhibits features due to nAChR-bound Carb (*short dashed lines*), the formation of physical interactions between Carb and nAChR binding site residues, and the Carb-induced resting-to-desensitized conformational change (*asterisks*). A signature spectrum of Carb is found in Baenziger *et al.* (13). The difference between spectra of the nAChR recorded in the presence and absence of 200 μM dibucaine (*middle panel on left and middle spectrum on right*) exhibit similar features indicative of dibucaine-induced structural change in the nAChR, but these are masked by the strong absorption bands of dibucaine partitioned into the lipid bilayer (*long dashed lines*) as well as the intense negative protein and lipid bands that reflect expansion of the nAChR film beyond the penetration depth of the infrared light from the germanium internal reflection element. At 200 μM concentrations, dibucaine binds to both the two neurotransmitter and NCB sites in the ion channel pore. A signature spectrum of dibucaine is found in Ryan and Baenziger (12). To avoid the spectral changes associated with partitioning into the lipid bilayer, the difference between spectra of the nAChR are recorded in the presence and absence of Carb, but while continuously maintaining the nAChR in contact with 200 μM dibucaine (*bottom panel on left and bottom spectrum on right*). The resulting difference spectrum exhibits positive and negative features due to the binding of Carb to and consequent displacement of dibucaine from the neurotransmitter site, respectively (*short and long dashed lines, respectively*). Bands indicative of the resting-to-desensitized conformational change noted with *asterisks* in the *top Carb difference spectrum* are absent because dibucaine stabilizes the nAChR in a desensitized state prior to the addition of Carb. Note that the Carb vibration near 1720 cm^{-1} (*dashed line on far left*) in both the *top and bottom spectrum* has an intensity of roughly 7.5×10^{-5} absorbance units while the negative lipid vibration near 1740 cm^{-1} in the *dibucaine difference spectrum* has a negative intensity of roughly 2.5×10^{-4} absorbance units.

trace). Although the resulting difference spectra do not provide a direct map of the NCB-induced structural change, they are informative because NCBs influence both the equilibrium between the resting and desensitized states and in turn the affinity of the nAChR for Carb. As Carb difference spectra exhibit bands that reflect both the resting-to-desensitized conformational change and the formation of physical interactions between Carb and the nAChR, NCB-induced variations in Carb difference band intensity provide insight into the structural basis of NCB action at the nAChR.

Proadifen—Carb difference spectra recorded in the presence of proadifen exhibit changes in the intensities of a number of difference bands that reflect structural consequences of proadifen binding to the nAChR. In particular, proadifen leads to dose-dependent decreases in the intensities of five bands, centered near 1663, 1655, 1547, 1430, and 1059 cm^{-1} , which serve as markers of the resting-to-desensitized conformational transition (12, 14). Bands centered near 1663 and 1655 cm^{-1} are in the amide I region (predominantly peptide C=O stretch) and are coupled to the 1547 cm^{-1} amide II vibration (predominantly peptide N-H bend). All three likely reflect a conformational change in the polypeptide backbone upon desensitization. The bands centered near 1430 (not shown) and 1059 cm^{-1} remain to be assigned, but likely reflect a structural change in individual side chains. Spectral changes that occur at proadifen concen-

trations up to 50 μM , where binding is restricted to the NCB site ($K_d \sim 3 \mu\text{M}$), are presented in Fig. 2. Those that occur at proadifen concentrations between 100 and 400 μM Carb, where additional binding occurs at the neurotransmitter site ($K_d \sim 400 \mu\text{M}$), are presented in Fig. 3 (17). The dose-dependent spectral changes are summarized in Fig. 6.

The binding of proadifen to the NCB site leads to a loss of intensity in three of the five conformationally sensitive bands centered near 1663, 1547, and 1059 cm^{-1} (Fig. 2). The loss of intensity near 1663 is best monitored as a change in the 1668/1680 cm^{-1} intensity ratio (see "Experimental Procedures"). Both the decrease in the 1668/1680 cm^{-1} ratio and loss of intensity centered near 1059 cm^{-1} are maximal at 50 μM proadifen, whereas additional changes in intensity near 1547 cm^{-1} are observed at higher proadifen concentrations (see below). The decrease in the 1668/1680 cm^{-1} ratio and loss of intensity centered near 1059 cm^{-1} indicate that the conformationally sensitive residues vibrating at 1663 and 1059 cm^{-1} are not able to undergo the resting-to-desensitized conformational change upon the binding of Carb. The simplest interpretation is that proadifen binding to the NCB site stabilizes the conformationally sensitive residues vibrating at these two frequencies in a desensitized conformation prior to the addition of Carb (see below).

In contrast, positive band intensity centered near 1655 and

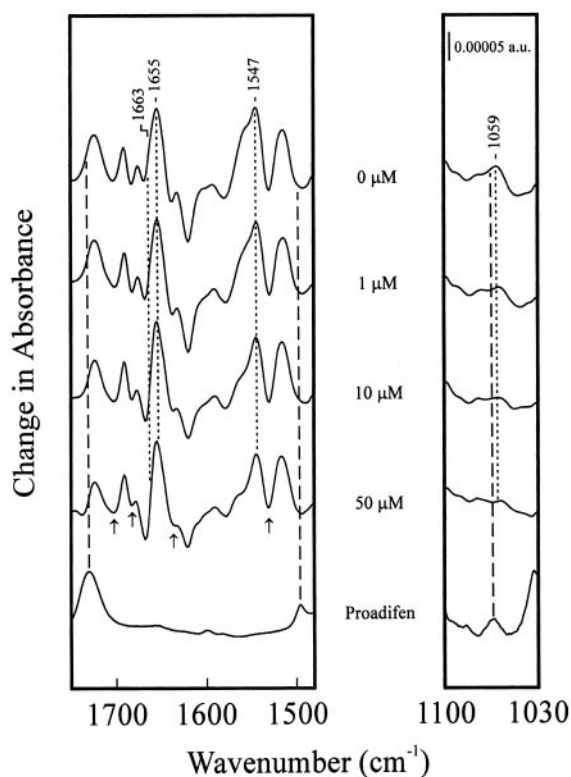


FIG. 2. Selected regions of Carb difference spectra recorded in the presence of the noted concentrations of proadifen. At these concentrations proadifen binding is restricted to the NCB site in the ion channel pore. The *short dashed lines* denote positive intensity in the difference spectra that reflects specifically the resting-to-desensitized conformational change (see text). *Long dashed lines* denote the frequencies at which proadifen itself absorbs infrared light. The *lowest trace* is an absorbance spectrum of proadifen recorded using the ATR technique in aqueous solution. The absorbance due to water has been subtracted. The *bar at top right* denotes the absorbance scale for the Carb difference spectra. *Arrows on the second trace from bottom* designate frequencies that are typically at or close to the base line.

1430 cm^{-1} (latter not shown) is essentially unaffected by concentrations of proadifen up to $50\text{ }\mu\text{M}$. A change in intensity at 1655 cm^{-1} is best monitored as a change in the $1655/1637\text{ cm}^{-1}$ ratio. At $50\text{ }\mu\text{M}$ proadifen, the conformationally active residues that vibrate near 1655 and 1430 cm^{-1} retain the ability to undergo a Carb-induced resting-to-desensitized conformational change and must therefore remain in a resting-like conformation despite the presence of bound proadifen. The contrasting effects of proadifen on the intensities of the conformationally sensitive bands near $1663/1059\text{ cm}^{-1}$ and $1655/1430\text{ cm}^{-1}$ suggest that the binding of proadifen to the NCB site shifts some nAChR residues into a desensitized-like conformation while others remain in a resting-like state. Proadifen binding to the NCB site thus leads to the formation of a conformation of the nAChR that is an intermediate between the resting and desensitized states and that shares structural features in common with both conformations.

Higher concentrations of proadifen lead to additional changes in the Carb difference spectra, although these are slightly distorted by a broad positive artifact between 1700 and 1500 cm^{-1} due to increasing instability of the nAChR film at higher proadifen concentrations (Fig. 3). Regardless of these distortions, it is clear that concentrations of proadifen between 100 and $400\text{ }\mu\text{M}$ lead to a substantial decrease in the $1655/1637\text{ cm}^{-1}$ ratio and a loss of intensity near 1547 and 1430 cm^{-1} . These intensity changes are most easily visualized upon superimposition of the difference spectra recorded at 100 and $400\text{ }\mu\text{M}$ proadifen (Fig. 3, see *dashed line spectrum*). No additional

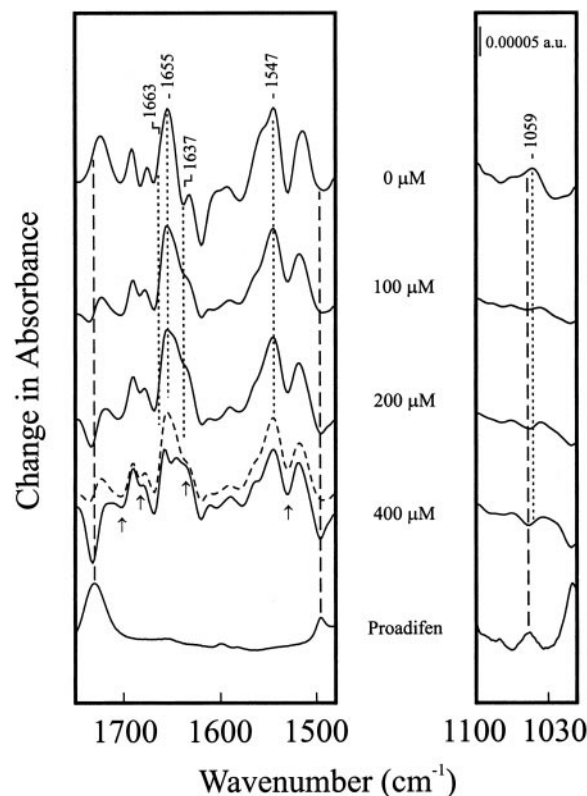


FIG. 3. Selected regions of Carb difference spectra recorded in the presence of the noted concentrations of proadifen consistent with proadifen binding to both the NCB and neurotransmitter sites. The *short dashed lines* denote positive intensity in the difference spectra that reflects specifically the resting-to-desensitized conformational change (see text). *Long dashed lines* denote the frequencies at which proadifen itself absorbs infrared light. The negative features at these frequencies in the difference spectra corresponds to the binding of proadifen to the neurotransmitter site (see text and *bottom schematic* in Fig. 1). The *lowest trace* is an absorbance spectrum of proadifen as in Fig. 2. *Arrows on the second trace from bottom* designate frequencies which are typically at or close to the base line, but are distorted to follow a rainbow-like pattern between 1700 and 1500 cm^{-1} because of nAChR film instability at high concentrations of proadifen (see "Experimental Procedures"). The Carb difference spectrum recorded at $100\text{ }\mu\text{M}$ proadifen (*dashed line*) is superimposed with a slight *y* axis offset on the Carb difference spectrum recorded at $400\text{ }\mu\text{M}$ proadifen (*solid line, second trace from bottom*).

intensity changes were consistently detected near either 1663 or 1059 cm^{-1} .

The decrease in the $1655/1637\text{ cm}^{-1}$ ratio and loss of intensity near 1547 and 1430 cm^{-1} can be attributed to changes in nAChR structure resulting from proadifen binding to the neurotransmitter site. This interpretation is based on the known K_d of proadifen for the neurotransmitter site (17) as well as the appearance of negative band intensity in the difference spectra (Fig. 3, *long dashed lines*) at frequencies that match the molecular vibrations of proadifen itself. These negative bands reflect the competitive displacement of proadifen from the neurotransmitter site upon the addition of Carb (Fig. 1, see *bottom schematic*). In addition, there appear to be subtle changes in the intensities of two bands near 1620 and 1516 cm^{-1} . The latter spectral changes likely result from an ability of proadifen to mimic some of the physical interactions that normally occur between Carb and neurotransmitter binding site residues (12). The absence of positive intensity at the five noted conformationally sensitive frequencies centered near 1663 , 1655 , 1547 , 1430 , and 1059 cm^{-1} in Carb difference spectra recorded at elevated concentrations of proadifen indicates that the additional binding of proadifen to the neurotransmitter site com-

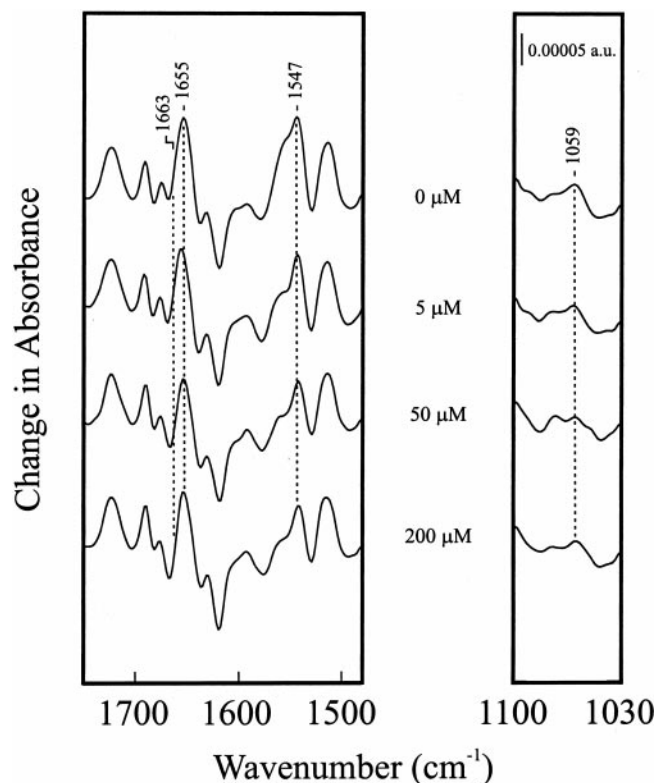


FIG. 4. Selected regions of Carb difference spectra recorded in the presence of the noted concentrations of PCP consistent with PCP-binding to the NCB site in the ion channel pore. The short dashed lines denote positive intensity in the difference spectra that reflects specifically the resting-to-desensitized conformational change (see text).

pletely abolishes the ability of the nAChR to undergo the Carb-induced resting-to-desensitized conformational transition. In agreement with data obtained for several other NCBs (12), the binding of proadifen to the neurotransmitter site thus stabilizes the nAChR in a fully desensitized state.

Note that the proadifen-sensitive vibration near 1547 cm^{-1} is a multicomponent band that is coupled to the two vibrations near 1663 and 1655 cm^{-1} . The coupling of these vibrations explains why changes in nAChR structure reflected by a loss of intensity near 1547 cm^{-1} occur as a consequence of proadifen binding to both the NCB and neurotransmitter binding sites.

PCP—Additional evidence for the existence of a conformational intermediate between the resting and desensitized states of the nAChR was obtained from both Carb difference spectra recorded in the presence of PCP and direct PCP difference spectra. PCP binds to the pore of the ion channel with dissociation constants of $1\text{--}6\text{ }\mu\text{M}$, whereas binding to the neurotransmitter site is relatively weak ($K_d \sim 250\text{ }\mu\text{M}$) (9, 18).

Carb difference spectra recorded while maintaining the nAChR in continuous contact with PCP at concentrations consistent with NCB site binding exhibit the same spectral changes observed upon proadifen binding to the NCB site, although the changes are less pronounced (Fig. 4). The presence of PCP reduces both the $1668/1680\text{ cm}^{-1}$ ratio and the positive band intensity near 1547 and 1059 cm^{-1} , but has no effect on the $1655/1637\text{ cm}^{-1}$ ratio or the positive band intensity near 1430 cm^{-1} (Fig. 6). As with proadifen, PCP binding to the NCB site stabilizes the conformationally sensitive residues that vibrate near 1663 and 1059 cm^{-1} in a desensitized-like state, whereas those that vibrate near 1655 and 1430 cm^{-1} remain in a resting-like conformation. Note that the PCP-induced changes in intensity near 1663 and 1059 cm^{-1} are, as

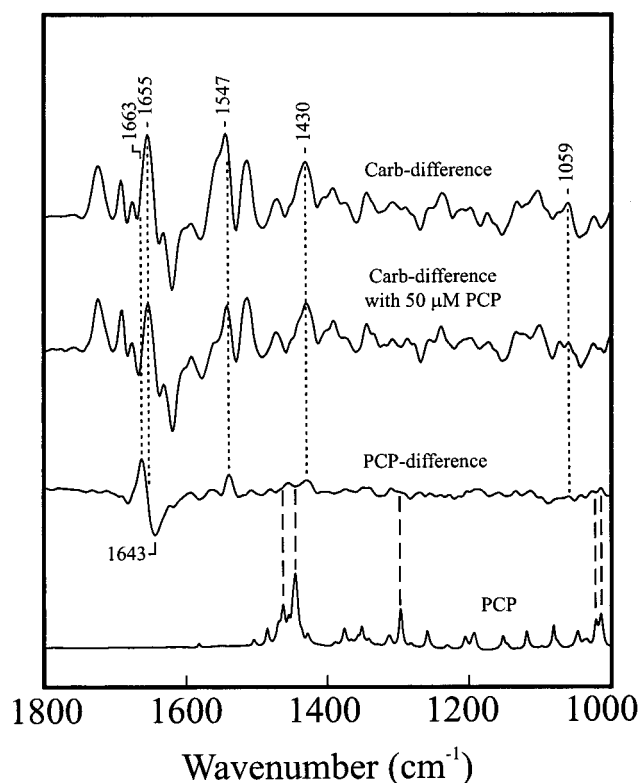


FIG. 5. Comparison of Carb difference spectra recorded in the presence and absence of $50\text{ }\mu\text{M}$ PCP with a PCP difference spectrum. The top trace is a Carb difference spectrum recorded in the absence of NCB (Fig. 1, top schematic). The second trace from top is a Carb difference spectrum recorded in the continuous presence of $50\text{ }\mu\text{M}$ PCP (Fig. 1, bottom schematic). The second trace from bottom is a PCP difference spectrum (Fig. 1, middle schematic). The bottom trace is an absorbance spectrum of powdered PCP recorded using the ATR technique. Hydration likely leads to a broadening of the absorbance bands in the PCP absorbance spectrum. The short dashed lines denote the bands that reflect the resting-to-desensitized conformational change.

expected, small compared with those induced by proadifen (Fig. 2). In contrast to proadifen, saturation of PCP binding to the NCB site leads to only a modest shift of the nAChR into a high affinity binding conformation. The relatively minor effects of PCP on both the Carb difference spectra and nAChR agonist binding affinity is likely a consequence of the only slight preference of PCP for the desensitized ($K_d \sim 1\text{ }\mu\text{M}$) versus resting ($K_d \sim 6\text{ }\mu\text{M}$) states (9, 18).

The difference between spectra of the nAChR recorded in the presence and absence of PCP, referred to as a PCP difference spectrum, probes directly the vibrational/structural changes that occur upon PCP binding to the nAChR (Fig. 1, middle schematic). PCP difference spectra exhibit three relatively intense bands located near 1663 , 1643 , and 1547 cm^{-1} , as well as several less intense bands in the $1500\text{--}1000\text{ cm}^{-1}$ region (Fig. 5, second trace from bottom). The latter include vibrations due to nAChR-bound PCP and are difficult to accurately assign due to limited signal-to-noise. The negative and a positive amide I difference bands centered near 1643 cm^{-1} and 1663 cm^{-1} , respectively, likely result from a shift in an amide I vibration of one or more residues from 1643 in the absence of PCP to 1663 cm^{-1} in the PCP-bound state. Both vibrations are likely coupled to the amide II difference band near 1547 cm^{-1} and likely reflect a PCP-induced change in the conformation of the polypeptide backbone.

The appearance of a strong positive difference band near 1663 cm^{-1} in the PCP difference spectrum is consistent with the loss of band intensity near 1663 cm^{-1} in Carb difference

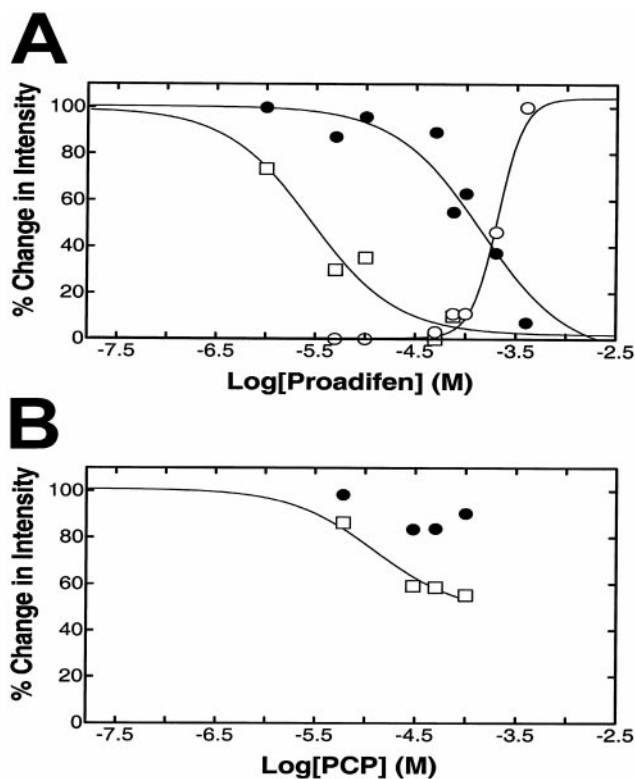


FIG. 6. Dose-dependent effects of proadifen and PCP on the intensities of selected bands in the Carb difference spectra. *A*, dose-dependent effects of proadifen on the 1668/1680 cm^{-1} intensity ratio (\square), 1655/1637 cm^{-1} intensity ratio (\bullet) and the intensity centered near 1740 cm^{-1} (\circ). The appearance of negative intensity at 1740 cm^{-1} reflects the binding of proadifen to the neurotransmitter site and its subsequent displacement upon the binding of Carb. The proadifen-induced concentration-dependent changes in intensity were curve-fitted using nonlinear least-squares resulting in EC_{50} values for the 1668/1680 cm^{-1} ratio and 1655/1637 cm^{-1} ratio of 3.2 and 140 μM , respectively. These values correspond to binding to the NCB and neurotransmitter sites, respectively (EC_{50} for 1740 cm^{-1} is 210 μM). *B*, dose-dependent effects of PCP on the 1668/1680 cm^{-1} ratio (\square) and 1655/1637 cm^{-1} ratio (\bullet). Vibrations due to PCP itself cannot be detected in the Carb difference spectra. The calculated EC_{50} value for the 1663 cm^{-1} band intensity change in the presence of PCP is 12 μM .

spectra recorded in the *continuous* presence of PCP (Fig. 5). The lack of an intense band near 1655 cm^{-1} in the PCP difference spectrum is also consistent with the lack of a substantial intensity change in this region of the Carb difference spectra (Fig. 6). These data conclusively show that PCP binding to the NCB site leads to a subtle conformational change from the resting state. It can thus be concluded that the conformationally sensitive residues that vibrate at 1663 cm^{-1} in the Carb difference spectrum are stabilized in a desensitized-like conformation upon PCP binding to the NCB site.

Effect of PCP on [^{125}I]TID Labeling of the Resting nAChR Channel—The conformational changes in the nAChR elicited by NCB binding to the ion channel pore were investigated further using the uncharged photoreactive compound [^{125}I]TID. [^{125}I]TID is a potent NCB of the nAChR, binding with micromolar affinity to both the resting and desensitized state of the receptor (18, 19). In the resting state, [^{125}I]TID specifically photolabels homologous aliphatic residues at positions 9 and 13 in each channel-lining M2 segment (e.g. $\delta\text{Leu-265}$ and $\delta\text{Val-269}$; Ref. 20). While TID binds with equal affinity to both the resting and desensitized channel, [^{125}I]TID photoincorporates into the resting channel ~ 10 -fold more efficiently than into the desensitized channel. In the absence of agonist the vast majority of [^{125}I]TID incorporation into individual receptor subunits reflects labeling of the resting channel. The

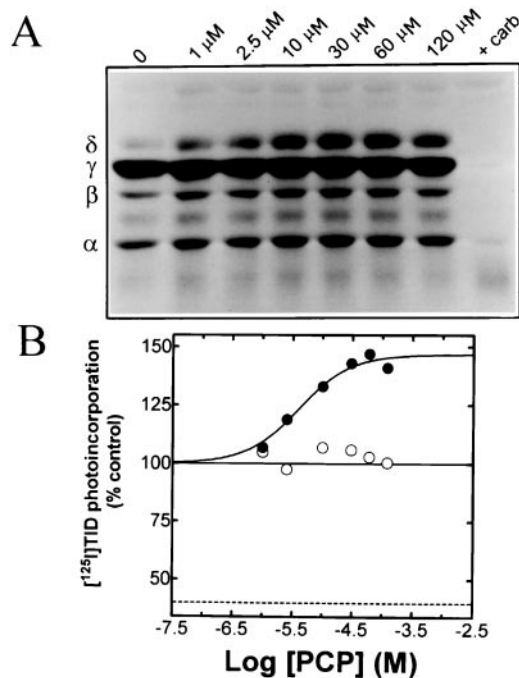


FIG. 7. Effects of PCP on the photoincorporation of [^{125}I]TID into the nAChR channel in the resting state. nAChR-rich membranes were equilibrated (2 h) with [^{125}I]TID (0.4 μM) in the absence (lanes 1–7) and in the presence (lane 8) of 400 μM Carb or in the presence of increasing concentrations of PCP (lanes 2–7). nAChR-rich membranes were then irradiated at 365 nm for 7 min, and polypeptides resolved by SDS-PAGE. *A*, shown is the corresponding autoradiograph of the gel containing the concentration-response labeling experiments for [^{125}I]TID *versus* PCP. The positions of the nAChR subunits are indicated on the left. *B*, for each concentration of PCP, individual nAChR subunit bands were excised from the dried gel and the amount of [^{125}I]TID photoincorporated into each subunit determined by γ counting. The amount of [^{125}I]TID subunit incorporation (\bullet , \circ) determined for each concentration of PCP is expressed as a percentage of the [^{125}I]TID subunit incorporation detected in the absence of any ligand. No significant ($>10\%$) concentration-dependent difference in the extent of [^{125}I]TID incorporation was detected in the α -, γ -, or β -subunit (β -subunit (\circ)). The concentration-dependent increase in [^{125}I]TID incorporation into the δ -subunit (\bullet) was curve-fitted using nonlinear least-squares resulting in a calculated EC_{50} value of 3.9 μM and a maximal increase of 146%. The dashed line indicates the amount of [^{125}I]TID incorporation into the δ -subunit in the presence of agonist and defines the level of nonspecific labeling.

addition of nAChR agonist or NCBs such as tetracaine reduces by greater than 75% the incorporation of [^{125}I]TID into receptor subunits (20, 21).

To assay the effect of both proadifen and PCP on [^{125}I]TID incorporation into the resting nAChR, nAChR-rich membranes were equilibrated in the absence of agonist, with [^{125}I]TID and increasing concentrations of both NCBs. Following irradiation and SDS-PAGE, all four nAChR subunits were efficiently labeled in the absence of NCB with 4-fold greater labeling of the γ -subunit compared with that of α -, β -, or δ -subunits (Fig. 7A, lane 1). Addition of agonist alone (400 μM carbamylcholine) decreased the extent of incorporation of [^{125}I]TID into each nAChR subunit by 75% or greater (93% for γ -subunit; Fig. 7A, lane 8). Equilibration of the resting state nAChR with proadifen led to a complete loss of [^{125}I]TID labeling (data not shown) likely due to competitive displacement of [^{125}I]TID from the ion channel pore, as has been observed for other NCBs (21). In contrast, PCP had no detectable, concentration-dependent effect on the extent of [^{125}I]TID incorporation into either the α -, β -, or γ -subunits (Fig. 7A), but led to a concentration-dependent increase in the extent of [^{125}I]TID incorporation into the δ -subunit. The concentration dependence of the increase in [^{125}I]TID

labeling by PCP was quantified by excising the δ -subunit bands from the dried polyacrylamide gel and determining the amount of ^{125}I present by γ -counting. The amount of cpm (^{125}I)TID associated with the δ -subunit band for each concentration of PCP was plotted as a percentage of the incorporation detected in the δ -subunit in the absence of any ligand. For the data presented in Fig. 7B, an EC_{50} value of $3.9\ \mu\text{M}$ was calculated for the increase in [^{125}I]TID incorporation into the δ -subunit by PCP with a maximal increase of 146%. Preincubation of the nAChR with α -bungarotoxin, which should prevent PCP binding to the agonist recognition site, had no detectable effect on the ability of PCP to increase the extent of [^{125}I]TID incorporation into the δ -subunit. Further, substitution of nAChR-rich membranes with affinity-purified nAChRs resulted in qualitatively similar results for the PCP-induced increase in [^{125}I]TID incorporation into the δ -subunit.

DISCUSSION

Numerous studies have shown that a variety of NCBs modulate nAChR conformational equilibria by binding to sites within the ion channel pore. Proadifen and PCP, etc., bind to histrionicotoxin-sensitive sites in the ion channel and stabilize a high affinity agonist binding conformation. Others, such as chlorpromazine and dimethisoquin, bind to histrionicotoxin-insensitive sites in the ion pore with similar effects on agonist binding affinity. In contrast, binding of the NCB tetracaine to the ion channel leads to the formation of a low affinity agonist binding state. Most blockers also bind to the neurotransmitter sites as well as to numerous low affinity sites on the nAChR with unclear effects on agonist binding affinity.

The changes in agonist binding affinity elicited upon NCB binding to the ion channel pore are usually interpreted in terms of a two-state conformational model. This model is based on the fundamental assertion that in the absence of bound ligands the nAChR exists in equilibrium between two conformations, a low affinity agonist binding resting and a high affinity agonist binding desensitized state. The equilibrium between these two strongly favors the resting state with only ~20% of the nAChRs adopting a desensitized conformation (1–3). Blockers such as proadifen, PCP, chlorpromazine, and dimethisoquin, etc., which stabilize a high affinity agonist binding conformation, are thought to shift the equilibrium in favor of the desensitized conformation (9, 10). Conversely, those such as tetracaine, which stabilize a low affinity agonist binding conformation, are thought to shift the equilibrium in favor of the resting state (11).

The data presented here indicate a more complex model of NCB action and illustrate a rich conformational diversity of the nAChR (Fig. 8). As a starting point for the interpretation of our data, we have also made the assumption that in the absence of bound ligand the nAChR exists in an equilibrium between two conformations, the resting and desensitized states. Our data indicate that “desensitizing” NCBs bind to the ion channel pore and stabilize a conformation intermediate between the resting and desensitized states that must, based on previous studies (9, 10), bind acetylcholine with a high affinity. In contrast, sensitizing NCBs bind to the ion channel pore and stabilize a complementary intermediate between the resting and desensitized states that binds acetylcholine with low affinity. In addition, the binding of NCBs to the neurotransmitter site leads to the formation of a fully desensitized state, regardless of whether or not NCB-site binding favors low or high affinity agonist binding conformations.

Our modified model of NCB action is based on the following observations. First, we have identified five positive bands near 1663, 1655, 1547, 1430, and 1059 cm^{-1} in Carb difference spectra that reflect the vibrational changes in the nAChR that

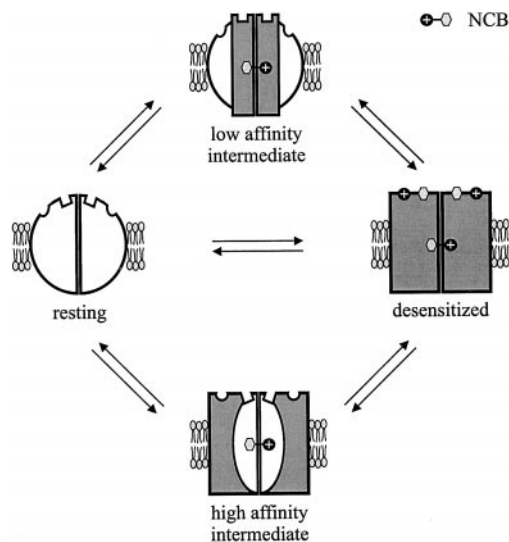


FIG. 8. Schematic model of the conformational effects of NCB binding to the nAChR. In the absence of bound ligand, nAChR exists in an equilibrium between the low affinity resting and high affinity desensitized states. “Desensitizing” NCBs bind to the ion channel pore and stabilize a high affinity conformation intermediate between the resting and desensitized states. “Sensitizing” NCBs bind to the ion channel pore and stabilize a low affinity intermediate between the resting and desensitized states. The binding of NCBs to the neurotransmitter site leads to the formation of a fully desensitized state.

are associated specifically with the resting-to-desensitized conformational transition (6, 12, 14). Carb difference spectra recorded in the presence of proadifen lose intensity at all five frequencies, an indicator that the nAChR has adopted a desensitized conformation, only at concentrations consistent with binding to the neurotransmitter sites and where spectral features indicative of neurotransmitter site binding are observed (see “Results”). Similarly, spectral changes indicative of full desensitization are only observed upon the binding of the NCBs dibucaine, prilocaine, and lidocaine to the neurotransmitter binding sites (12). In addition, the binding of tetracaine to the neurotransmitter site leads to spectral changes indicative of desensitization, despite the fact that tetracaine binding to the ion channel pore stabilizes a low as opposed to a high affinity agonist binding conformation (12). These results illustrate that neurotransmitter site binding is not only required but is alone sufficient for NCBs to stabilize a fully desensitized nAChR.

Second, Carb difference spectra recorded in the presence of concentrations of proadifen, and PCP consistent with binding exclusively to the NCB site exhibit a loss in the intensities of only two of the noted conformationally sensitive bands centered near 1663 and 1059 cm^{-1} , while the two conformationally sensitive vibrations centered near 1655 and 1430 cm^{-1} are essentially unaffected. The loss of intensity at both 1663 and 1430 cm^{-1} indicates that the residues giving rise to these bands do not undergo the resting-to-desensitized conformational change upon Carb binding and are likely stabilized in a desensitized conformation prior to the addition of Carb. In contrast, the lack of a change in intensity near 1655 and 1430 cm^{-1} indicates that the residues giving rise to the latter bands remain in a resting-like conformation that can still respond to Carb binding. PCP difference spectra, which probe directly the PCP-induced conformational change, exhibit positive intensity near 1663 cm^{-1} and possibly 1059 cm^{-1} with no change in intensity near 1655 cm^{-1} , confirming that NCB site binding leads to a structural change in only those residues that vibrate at the former two frequencies from the resting to a desensitized-like conformation. In addition, similar patterns of band intensity changes are observed in Carb difference spectra re-

corded at concentrations of prilocaine and lidocaine consistent with NCB site binding, although the overlapping affinities of these two blockers for the NCB and neurotransmitter sites make the data difficult to interpret (12). Collectively, these results conclusively show that NCB site binding leads to the formation of a conformation that is a structural intermediate between the resting and desensitized states. In this structural intermediate, some residues remain in a resting-like while others adopt a desensitized-like conformation. This intermediate is referred to as the high affinity intermediate.

Third, Carb difference spectra recorded in the presence of the sensitizing NCB tetracaine at concentrations consistent with binding to the NCB site exhibit an increase in intensity centered near 1663 and 1059 cm^{-1} with little or no effect on band intensity near 1655 and 1430 cm^{-1} (12). Tetracaine binding to the NCB site thus influences the same residues that are affected by proadifen and PCP binding to the NCB site binding, but in contrast the data suggest a shift in these residues from a desensitized-like to a resting-like conformation. This interpretation is consistent with the decrease, as opposed to an increase in agonist binding affinity observed upon tetracaine binding to the NCB site. It can thus be concluded that tetracaine stabilizes an intermediate between the resting and desensitized states that is complimentary to that stabilized by either proadifen or PCP and that binds acetylcholine with a low affinity. This intermediate is referred to as the low affinity intermediate.

Finally, the [^{125}I]TID labeling pattern of the nAChR in the presence of PCP is distinct from the [^{125}I]TID labeling pattern observed in either the presence (desensitized conformation) or absence (predominantly resting conformation) of Carb. Although inconclusive with respect to the formation of a conformational intermediate in response to PCP binding, the [^{125}I]TID labeling suggest the formation of a conformation that is distinct from both the resting and desensitized states and is thus consistent with the FTIR data. Note also that a similar labeling pattern has been detected in the presence of ketamine, memantine, and amantadine.²

The observation that both proadifen and PCP stabilize a conformation intermediate between the resting and desensitized states has important implications. Numerous studies have shown that the binding of either NCB to the ion channel leads to the formation of a conformation that has a high affinity for acetylcholine. In fact, increased acetylcholine binding affinity is typically used as an indicator of desensitization. Our data show that the high affinity conformation stabilized by proadifen and PCP is an intermediate between the resting and desensitized states. It can thus be concluded that an increase in acetylcholine binding affinity is not sufficient to define the desensitized conformation. More comprehensive methods are required to define the conformation status of the nAChR.

The Carb difference spectra recorded in the presence of PCP and proadifen also show that regions of the nAChR inter-convert between the resting and desensitized conformations independently of

each other. Although it is tempting to suggest that this independent conformational change of domains leads to an uncoupling of the transmembrane domain from the extramembranous neurotransmitter binding site, the fact that proadifen and PCP binding to the ion channel alters both agonist binding affinity indicates that this is not the case (see also Ref. 22). In this light it is interesting to note that a recent linear free energy analysis of nAChR channel gating suggested that the binding affinity of the nAChR for acetylcholine increases along the pathway between agonist binding and the open state (23). The conformational intermediate detected here upon NCB binding to the ion channel pore may share structural similarities to this transient conformational intermediate.

Finally, it should be noted that the high affinity structural intermediate between the resting and desensitized states detected in this study is also detected in Carb difference spectra recorded from the nAChR reconstituted into egg phosphatidylcholine membranes containing small amounts of either phosphatidic acid or cholesterol. A structural intermediate between the resting and desensitized states may therefore exist under a variety of physiological conditions, in both the absence and presence of bound ligands. Conformational diversity may play a role in the modulation of the post-synaptic response by both endogenous and exogenous factors. Further studies are required to define the biophysical properties of this intermediate to understand its possible biological significance.

REFERENCES

1. Heidmann, T., and Changeux, J.-P. (1979) *Eur. J. Biochem.* **94**, 255–279
2. Boyd, N. D., and Cohen, J. B. (1980) *Biochemistry* **19**, 5334–5353
3. Boyd, N. D., and Cohen, J. B. (1980) *Biochemistry* **19**, 5353–5358
4. Haganir, R. L., Delcour, A. H., Greengard, P., and Hess, G. P. (1986) *Nature* **321**, 774–776
5. Swope S. L., Moss, S. I., Raymond, L. A., and Haganir, R. L. (1999) *Adv. Second Messenger Phosphoprotein Res.* **33**, 49–78
6. Baenziger, J. E., Morris, M.-L., Darsaut, T. E., and Ryan, S. E. (2000) *J. Biol. Chem.* **275**, 777–784
7. Heidmann, T., and Changeux, J.-P. (1978) *Annu. Rev. Biochem.* **47**, 371–411
8. Arias, H. R. (1996) *Mol. Membr. Biol.* **13**, 1–17
9. Heidmann, T., Oswald, R. E., and Changeux, J.-P. (1983) *Biochemistry* **22**, 3112–3127
10. Krodel, E. K., Beckman, R. A., and Cohen, J. B. (1979) *Mol. Pharmacol.* **15**, 294–312
11. Boyd, N. D., and Cohen, J. B. (1984) *Biochemistry* **23**, 4023–4033
12. Ryan, S. E., and Baenziger, J. E. (1999) *Mol. Pharmacol.* **55**, 348–355
13. Baenziger, J. E., Miller, K. W., and Rothschild, K. J. (1993) *Biochemistry* **32**, 5448–5454
14. Ryan, S. E., Demers, C. N., Chew, J. P., and Baenziger, J. E. (1996) *J. Biol. Chem.* **271**, 24590–24597
15. Chiara, D. C., and Cohen, J. B. (1997) *J. Biol. Chem.* **272**, 32940–32950
16. McCarthy, M. P., and Moore, M. A. (1992) *J. Biol. Chem.* **267**, 7655–7663
17. Blanchard, S. G., Elliot, J., and Raferty, M. A. (1979) *Biochemistry* **18**, 5880–5885
18. White, B. H., Howard, S., Cohen, S. G., and Cohen, J. B. (1991) *J. Biol. Chem.* **266**, 21595–21607
19. Wu, G., Raines, D., and Miller, K. (1994) *Biochemistry* **33**, 15375–15381
20. White, B. H., and Cohen, J. B. (1991) *J. Biol. Chem.* **267**, 15770–15783
21. Moore, M. A., and McCarthy, M. P. (1994) *Biochim. Biophys. Acta* **1190**, 457–464
22. Galzi, J. L., Revah, F., Bouet, F., Menez, A., Goeldner, M., Hirth, C., and Changeux, J. P. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 5051–5055
23. Grosman, C., Zhou, M., and Auerbach, A. (2000) *Nature* **403**, 773–776
24. Ryan, S. E., Nguyen, H. P., and Baenziger, J. E. (1998) *Toxicol. Lett.* **100–101**, 179–183
25. Baenziger, J. E., Miller, K. W., and Rothschild, K. J. (1992) *Biophys. J.* **61**, 983–992
26. Laemmli, U. K. (1970) *Nature* **227**, 680–685

² M. P. Blanton and E. A. McCarty, unpublished observations.