

A Structure-Based Approach to Nicotinic Receptor Pharmacology

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ABSTRACT

Infrared difference spectroscopy has been used to examine the structural effects of local anesthetic (LA) binding to the nicotinic acetylcholine receptor (nAChR). Several LAs induce subtle changes in the vibrational spectrum of the nAChR over a range of concentrations consistent with their reported nAChR-binding affinities. At concentrations of the desensitizing LAs prilocaine and lidocaine consistent with their binding to the ion channel pore, the vibrational changes suggest the stabilization of an intermediate conformation that shares structural features in common with both the resting and desensitized states. Higher concentrations of prilocaine and lidocaine, as well as the LA dibucaine, lead to additional binding to the neurotransmitter-binding site, the formation of physical interactions (most nota-

bly cation-tyrosine interactions) between LAs and neurotransmitter-binding-site residues, and the subsequent formation of a presumed desensitized nAChR. Although concentrations of the LA tetracaine consistent with binding to the ion channel pore elicit a reversed pattern of spectral changes suggestive of a resting state-like nAChR, higher concentrations also lead to neurotransmitter site binding and desensitization. Our results suggest that LAs stabilize multiple conformations of the nAChR by binding to at least two conformationally sensitive LA-binding sites. The spectra also reveal subtle differences in the strengths of the physical interactions that occur between LAs and binding-site residues. These differences correlate with LA potency at the nAChR.

Classical pharmacology has identified the sites of action for numerous agonists and antagonists on specific integral membrane receptors and has led to hypotheses regarding the molecular details of membrane receptor-drug interactions. Although site-directed mutagenesis has proven to be an effective method for testing these hypotheses by allowing the functional role of individual amino acid side chains to be assessed, the application of physical methods that can probe membrane receptor structure and conformational change at atomic resolution is required for a comprehensive understanding of drug action.

In the absence of high-resolution structural information, infrared difference spectroscopy has led to significant insight into the structural basis of integral membrane protein function and could, in principle, be used to probe structural aspects of membrane receptor-drug interactions. The infrared difference technique is capable both of detecting and elucidating subtle changes in structure that occur in individual amino acid residues upon protein conformational change but, for technical reasons, has been restricted mainly to studies of light-activated membrane proteins such as bacteriorhodopsin and the photosynthetic reaction center (Braiman and

Rothschild, 1988). Recently, the difference technique was modified for probing changes in structure that occur upon agonist binding to the nicotinic acetylcholine receptor (nAChR) from *Torpedo* (Baenziger et al., 1992, 1993). Here, we use this novel approach to investigate the mechanisms of local anesthetic (LA) action at the nAChR.

An important goal of this initial work was to investigate the utility of the difference technique for probing structural aspects of drug action at integral membrane receptors. The work focused on LA-nAChR interactions because the nAChR is a neurotransmitter-gated ion channel that is relatively well characterized in terms of its structure and function (Stroud et al., 1990; Galzi et al., 1991). The mechanisms of LA action at the nAChR are also relatively well understood, although not at the structural level. The current model suggests that most LAs bind to a noncompetitive blocker (NCB) site located within the ion channel pore, where they both block the conductance of cations across the membrane and stabilize a conformation that binds acetylcholine with high affinity (Krodel et al., 1979; Cohen et al., 1986). This high-affinity conformation is presumed to be analogous to the agonist-induced desensitized state (Boyd and Cohen, 1984). In contrast, other LAs compete for binding at the same NCB site but either have no effect or stabilize a conformation that binds acetylcholine with low affinity (Boyd and Cohen, 1984).

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Some LAs also modulate nAChR conformation by binding to the neurotransmitter and/or low-affinity sites on the nAChR (Heidmann et al., 1983).

We show here that the infrared difference technique can detect subtle LA-induced changes in the vibrational spectrum of the nAChR over concentration ranges consistent with known LA–nAChR-binding affinities. The data suggest that LA binding to the NCB site stabilizes a conformation of the nAChR that is structurally distinct from the conformation stabilized by LA binding to the neurotransmitter-binding site. The difference spectra also shed light on the nature of the physical interactions that occur between LAs and binding-site residues.

Materials and Methods

Sample Preparation. The nAChR from *Torpedo californica* was affinity-purified and reconstituted into a membrane composed of egg phosphatidylcholine/dioleoylphosphatidic acid/cholesterol in a lipid molar ratio of 3:1:1 (McCarthy and Moore, 1992). Aliquots containing 250 μ g of nAChR protein were deposited on the surface of a germanium internal reflection element. In each case, the excess buffer was evaporated with a gentle stream of N_2 gas. The nAChR film then was rehydrated with excess buffer (250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, and 20 mM Tris, pH 7.0) and placed in a thermostatically controlled attenuated total reflectance cell.

Carbamylcholine (Carb) Difference Spectra. All infrared spectra were acquired using the attenuated total reflectance technique (see schematic in Fig. 1A) on an FTS-40 spectrometer (Bio-Rad, Cambridge, MA) equipped with a DTGS detector (Ryan et al., 1996). Spectra were recorded at 8 cm^{-1} resolution using 512 scans each. This took roughly 7 min per spectrum. In general, two consecutive spectra were recorded while flowing buffer past the nAChR film surface (Fig. 1A, buffer i). The flowing solution was then switched to an identical buffer containing 50 μ M agonist Carb (Fig. 1A, buffer ii), and a spectrum was recorded of the desensitized state. The difference between the two resting-state spectra (both in the absence of Carb; control spectra) and the consecutive resting-state and desensitized-state (presence of Carb) spectra were calculated

and stored, and the flowing solution was 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.

The effects of LAs on the structures of those residues involved in ligand binding and desensitization were monitored by recording typical Carb difference spectra as described above but while continuously exposing the nAChR to a given concentration of LA (the LA was included in both the plus and minus Carb buffers; i.e., buffers i and ii in Fig. 1A). For each LA concentration, Carb difference spectra were recorded from a minimum of two different nAChR films (usually four or more) obtained from at least two separate reconstitutions. The presented difference spectra each represent the average of between 30 and 90 individual differences. LA-induced spectral changes were identified by superimposing normalized difference spectra on the computer. Spectra were normalized by comparing the intensity of bands that have been attributed to nAChR-bound Carb (denoted with asterisks in Fig. 2A) including those centered near 1720, 1480, and 1340 cm^{-1} . Because of the complexity of both the Carb difference spectra and the LA-induced spectral changes, the nature of the LA dose-response relationships was not examined graphically.

Results

The averaged difference between infrared spectra of the nAChR recorded while sequentially flowing buffer either with or without the agonist Carb past the nAChR film surface (see schematic in Fig. 1A) is referred to as a Carb difference spectrum and exhibits a highly reproducible pattern of positive and negative bands (Fig. 2, top trace in A–C). These positive and negative bands provide a spectral map of the structural changes that occur upon Carb binding to the nAChR (Baenziger et al., 1992, 1993). In particular, the difference bands represent vibrational changes associated with both the resting-to-desensitized conformational change and the formation of physical interactions, such as hydrogen bonds, cation- π electron interactions, etc., between Carb and neurotransmitter-binding site residues. Positive bands are

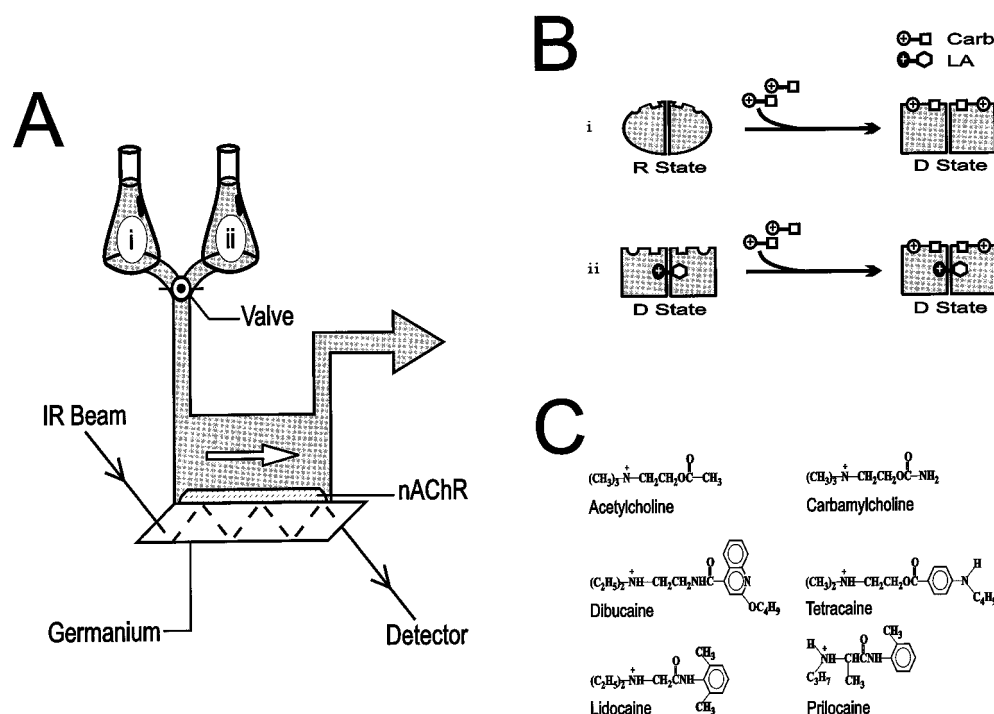


Fig. 1. A, schematic diagram of the attenuated total reflectance cell used for the acquisition of Carb difference spectra. For details see *Materials and Methods*. B, schematic representation of the conformational changes that are probed here using difference spectroscopy. i, A typical Carb difference experiment probes the conformational change between the resting and Carb-induced desensitized state. ii, Carb difference spectra recorded in the continuous presence of a LA probe the structural changes that occur upon Carb binding, except that the LA can stabilize an alternate conformation of the nAChR. For simplicity, scheme ii shows that the LA stabilizes a desensitized nAChR by binding exclusively to the NCB site within the ion channel pore. LAs may also bind to the neurotransmitter and/or low-affinity sites on the nAChR (see text). C, comparison of the structures of some simple LAs with acetylcholine and Carb.

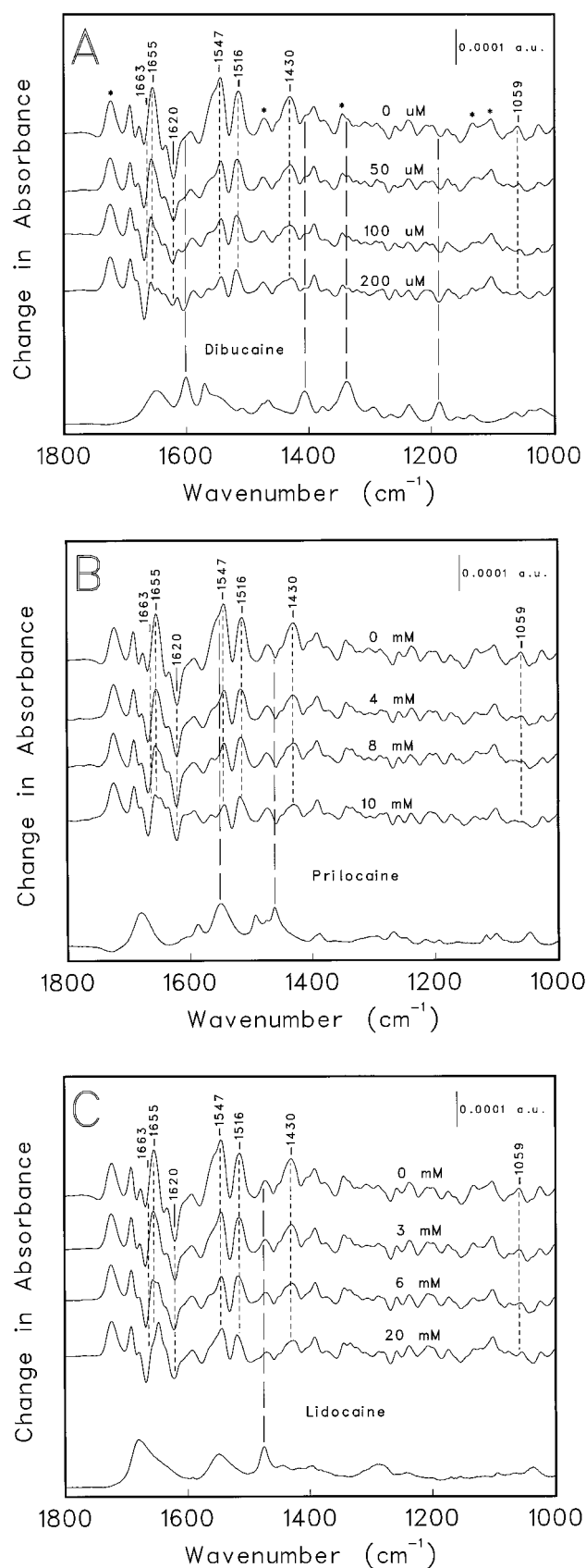


Fig. 2. Selected Carb difference spectra recorded in the presence of the noted concentrations of the LAs dibucaine (A), prilocaine (B), and lidocaine (C). The bottom trace in each is the absorbance spectrum recorded from a 50 mM solution of the respective LA (the buffer has been subtracted).

also observed in the Carb difference spectrum that reflect the vibrations of Carb bound specifically to the nAChR (Fig 2A, asterisks). A schematic diagram of the structural changes that are probed in a typical Carb difference spectrum is shown in Fig. 1B (scheme i). Note that in this report we refer to the difference between spectra of the nAChR recorded in the presence and absence of Carb as a Carb difference spectrum regardless of whether the nAChR undergoes the resting-to-desensitized conformational transition (see below).

The difference between infrared spectra of the nAChR recorded in the presence and absence of a LA similarly should exhibit spectral features that are indicative of LA-induced structural change. In some cases, however, these features are masked by relatively large intensity changes that result from the LA partitioning into, and causing the expansion of, the nAChR film on the surface of the germanium internal reflection element (data not shown). An alternative approach for monitoring LA-induced conformational change is to record typical Carb difference spectra while maintaining the nAChR in the continuous presence of a given concentration of LA. Variations in the pattern of bands observed in difference spectra recorded under these conditions should reflect LA-induced changes in the structure of those residues that are involved in the binding of Carb and subsequent desensitization. As LAs modulate the equilibrium between the resting and desensitized states and, in turn, influence the affinity of the nAChR for Carb, such spectral variations should provide insight into the structural basis of LA action at the nAChR. A schematic diagram of the conformational changes probed in a Carb difference spectrum recorded in the continuous presence of a LA is shown in Fig. 1B (scheme ii). For simplicity, this scheme assumes that the LA stabilizes a desensitized nAChR by binding exclusively to the NCB site. LAs also bind to the neurotransmitter and/or low-affinity sites as discussed below.

Desensitizing LAs. Carb difference spectra recorded in the presence of increasing concentrations of the desensitizing LAs dibucaine, prilocaine, and lidocaine exhibit variations in the intensity of a number of positive and negative difference bands (Fig. 2). These band-intensity variations occur over concentrations consistent with the known binding affinities of the LAs for the NCB- and/or neurotransmitter-binding sites and likely reflect structural changes that result specifically from LA binding to the nAChR (Table 1). The most notable variations include a marked decrease in the intensity of five positive bands centered near 1663 (see Fig. 3A), 1655, 1547, 1430, and 1059 cm^{-1} . Three of these five bands occur in either the amide I (1600–1700 cm^{-1}) or amide II (1520–1580 cm^{-1}) region and likely reflect a change in the conformation of the polypeptide backbone. The difference bands near 1430 and 1059 cm^{-1} likely reflect a change in the structure and/or environment surrounding individual amino acid side chains. Because all three LAs stabilize a desensitized nAChR, they should eliminate bands in the Carb difference spectrum that

The short, dashed lines mark the five bands that are indicative of the resting-to-desensitized conformational change (see text). The long, dashed lines illustrate the negative bands in the Carb difference spectra that reflect Carb-induced displacement of either dibucaine, prilocaine, or lidocaine from the nAChR. Asterisks in A (top trace) denote the vibrations of nAChR bound Carb (Baenziger et al., 1993). The bar represents a change in intensity in the difference spectra of 0.0001 absorbance units (a.u.).

result from the resting-to-desensitized conformational change itself (see scheme ii of Fig. 1B). The loss of intensity at each of these five frequencies could, therefore, reflect the formation of a desensitized nAChR. This possibility is supported by the observation of similar band intensity changes in difference spectra recorded from the nAChR reconstituted into egg phosphatidylcholine membranes, where the nAChR does not undergo agonist-induced conformational change (Ryan et al., 1996). In addition, the LA tetracaine, which stabilizes a resting-like conformation, leads to an increase as opposed to a decrease in intensity at each of these five frequencies (Fig. 3A). Note that weak protein vibrations underlying the 1720 cm^{-1} Carb vibration appear to be absent at most LA concentrations and could reflect changes in structure associated with the resting-to-desensitized conformational transition, although a rigorous analysis requires an agonist that does not absorb in this region.

The loss of band intensity indicative of the formation of a desensitized nAChR occurs for all three LAs concomitant with the appearance of negative bands at frequencies that match the vibrational frequencies of the LAs themselves. These negative features are clearly evident in Carb difference spectra recorded in the presence of dibucaine (Fig. 2A), but are only detected upon superimposition of the difference spectra recorded in the presence and absence of either prilocaine or lidocaine because of the extremely weak intrinsic absorbance intensity of the latter two LAs (not shown). The appearance of negative LA bands suggests that the addition of Carb leads to the displacement of each LA from the nAChR membrane film. The LAs are not likely displaced from the NCB site because Carb does not bind to this site at $50\text{ }\mu\text{M}$ concentrations. The affinities of all three LAs for the NCB site are also increased in the presence of Carb (Blanchard et al., 1979; Krodel et al., 1979). Control difference spectra recorded from α -bungarotoxin-treated nAChR membranes indicate that the majority of the negative LA intensity is neither a result of direct Carb/LA competition at a previously unidentified site distinct from either the neurotransmitter and/or NCB sites nor of a nonspecific Carb-induced displacement of the LAs from the lipid bilayer (data not shown). The negative LA vibrations therefore must reflect the competitive displacement of LAs from the neurotransmitter-binding site

by Carb. This interpretation is consistent with the known binding of all three LAs to the neurotransmitter-binding site over the studied ranges of LA concentrations (Table 1).

The LAs also have subtle effects on the intensity and possibly the frequency of difference bands that cannot be attributed to either the displacement of LAs from the nAChR or an effect on the equilibrium between the resting and desensitized states. In particular, dibucaine leads to a marked reduction in the intensity of the negative and positive difference bands located near 1620 and 1516 cm^{-1} , respectively. Lidocaine has a lesser effect on the intensity of both bands whereas prilocaine has no influence on the intensity near 1620 cm^{-1} but elicits a slight reduction in the intensity of the band centered near 1516 cm^{-1} . Note that neither the presence of tetracaine nor reconstitution of the nAChR into egg phosphatidylcholine membranes, both of which influence the conformational status of the nAChR, have marked effects on the intensity of either band (Fig. 4B; Ryan et al., 1996).

Vibrational bands in the Carb difference spectrum that are not due to either the resting-to-desensitized conformational change or the vibrations of nAChR-bound Carb must reflect the formation of physical interactions between Carb and neurotransmitter-binding site residues. The negative band near 1620 cm^{-1} has not yet been assigned to a particular amino acid side chain vibration (Baenziger and Chew, 1997). The 1516 cm^{-1} band, however, is highly characteristic of a ring-stretching vibration of tyrosine and likely reflects an increase in vibrational intensity associated with the formation of cation-tyrosine interactions between Carb and the nAChR (see *Discussion*). The LA-induced decrease in intensity of the 1516 cm^{-1} difference band suggests that LAs form similar cation-tyrosine interactions with the nAChR before the addition of Carb. In addition, the variable effects of the three LAs on the intensity of these two bands could indicate subtle differences in the ability of the LAs to mimic the binding of Carb to the neurotransmitter-binding site.

The concomitant appearance of spectral changes that are suggestive of the stabilization of a desensitized nAChR, the Carb-induced displacement of LAs from the neurotransmitter-binding site, and the formation of Carb-like physical interactions between LAs and neurotransmitter-binding site

TABLE 1

Comparison of both the binding affinities and conformational effects of the studied LAs at the NCB- and neurotransmitter-binding sites as determined by biochemical methods and infrared difference spectroscopy

Local Anesthetic	Literature				FTIR Data			
	NCB site		Neurotransmitter site		NCB site		Neurotransmitter site	
	C_{50}^a	Effect	C_{50}^b	Effect ^c	C_{max}^c	Effect	C_{max}^c	Effect
	μM		μM		μM		μM	
Dibucaine	80	D	56	N.D.	N.D.	N.D.	200	D
Lidocaine	2,500 (2,000) ^d	D	1,100	N.D.	3,000	I	~10,000	D
Prilocaine	300 (4,000) ^d	D	4,500	N.D.	4,000	I	10,000	D
Tetracaine	1.5	R	800	N.D.	10	I*	1,000	D

^a C_{50} represents the concentration of LA, in the absence of Carb, required to either reduce the specific binding of [^3H]perhydrohistrionicotoxin to the nAChR (dibucaine, lidocaine, and tetracaine) (Blanchard et al., 1979) or the concentration required to cause half the maximal observed increase in the binding of a fluorescent acetylcholine analog to the neurotransmitter-binding site (prilocaine) (Cohen et al., 1974).

^b C_{50} represents the concentration of LA required to reduce the initial rate of [α - ^{125}I]bungarotoxin (dibucaine, lidocaine, and tetracaine) or [^3H] α -toxin (prilocaine) by 50% (Blanchard et al., 1979; Weber and Changeux, 1974).

^c C_{max} represents the concentration of LA required to produce maximal changes in band intensity in the Carb difference spectra. D and R refer to the desensitized and resting conformations, respectively. The LAs dibucaine, prilocaine, and lidocaine are proposed to stabilize a conformation intermediate (I) between the R and D states upon binding to the NCB site that differs from the intermediate state (I*) stabilized by tetracaine (see text).

^d Values presented in parentheses represent the reported concentration of LA required to cause a maximal increase in fluorescent agonist binding to the neurotransmitter-binding site (Cohen et al., 1974).

^e N.D., no data.

residues imply that the main “desensitizing” effects of each LA occur as a consequence of binding to the neurotransmitter site. This interpretation contrasts with most models, which suggest that LAs stabilize a desensitized nAChR by binding to the NCB site, and questions whether the binding of LAs to the NCB site modulates the conformational equilibria of the nAChR. A close examination of the concentration dependencies of the spectral variations observed in the presence of prilocaine and lidocaine, however, reveals that there are structural changes that occur exclusively as a result of LA binding to the NCB site. Prilocaine and lidocaine at concentrations of 4 mM and 3 mM, respectively, elicit close to

maximal changes in the intensity of the two conformationally sensitive bands near 1663 and 1059 cm^{-1} , whereas the bands near 1655 and 1430 cm^{-1} are relatively unaffected (Fig. 4A). These LA concentrations essentially are equivalent to those at which prilocaine and lidocaine bind to the NCB site and lead to maximal effects on acetylcholine analog-binding affinity at the neurotransmitter-binding site (see Fig. 9 in Cohen et al., 1974). Although the overlapping affinities of prilocaine and lidocaine for the NCB- and neurotransmitter-binding sites prevent an unequivocal interpretation of the data, it appears that the binding of LAs to the NCB site causes those residues that vibrate near 1663 and 1059 cm^{-1} to adopt a structure similar to that found in the “desensitized” state whereas those residues that vibrate near 1655 and 1430 cm^{-1} retain a resting-like structure. LA binding to the NCB site may, therefore, stabilize a conformation that is a structural intermediate between the resting and what we have tentatively referred to as the desensitized state.

Tetracaine Binding to the NCB and Neurotransmitter Sites. The structural consequences of LA binding exclusively to the NCB- and neurotransmitter-binding sites were investigated further by recording Carb difference spectra in the presence of increasing concentrations of the LA tetracaine. Tetracaine has a more than 100-fold stronger affinity for the NCB site than the neurotransmitter-binding site. The binding of tetracaine to the NCB site stabilizes a conformation of the nAChR that binds acetylcholine with low as opposed to a high affinity. Tetracaine thus appears to shift the equilibrium between the resting and desensitized conformations toward the resting state (Boyd and Cohen, 1984). Note that within native membranes and in the absence of agonist, roughly 20% of the nAChR is found in the desensitized state.

Carb difference spectra recorded in the presence of up to 50 μM concentrations of tetracaine, where binding is restricted to the NCB site, exhibit changes in the intensity of the previously noted conformationally sensitive bands centered near 1663, 1655, 1547, 1430, and 1059 cm^{-1} , although, as expected, there is an increase as opposed to a decrease in the intensity of each band (Fig. 3A). Significantly, there is a relatively large increase in band intensity near 1663 and 1059 cm^{-1} compared with the intensity changes near 1655 and 1430 cm^{-1} (Figs. 3A and 4B). The binding of tetracaine to the NCB site thus appears to cause those residues that vibrate near 1663 and 1059 cm^{-1} to shift from a desensitized to a resting-like conformation whereas those residues that vibrate near 1655 and 1430 cm^{-1} mainly retain a desensitized-like structure. The binding of all three LAs to the NCB site thus appears to affect mainly those structures that vibrate near 1663 and 1059 cm^{-1} . Because tetracaine, prilocaine, and lidocaine allosterically influence acetylcholine-binding affinity by binding to the NCB site, it can be concluded that the changes in structure reflected by the two bands near 1663 and 1059 cm^{-1} lead to changes in acetylcholine-binding affinity, even though they may not constitute the formation of either a fully “desensitized” or sensitized nAChR (see *Discussion*). Note that there are no bands indicative of the Carb-induced displacement of tetracaine from the neurotransmitter-binding site at these concentrations. These low tetracaine concentrations also have minimal, if any, effects on the difference bands near 1620 and 1516 cm^{-1} .

In contrast, higher concentrations that result in the additional binding of tetracaine to the neurotransmitter-binding

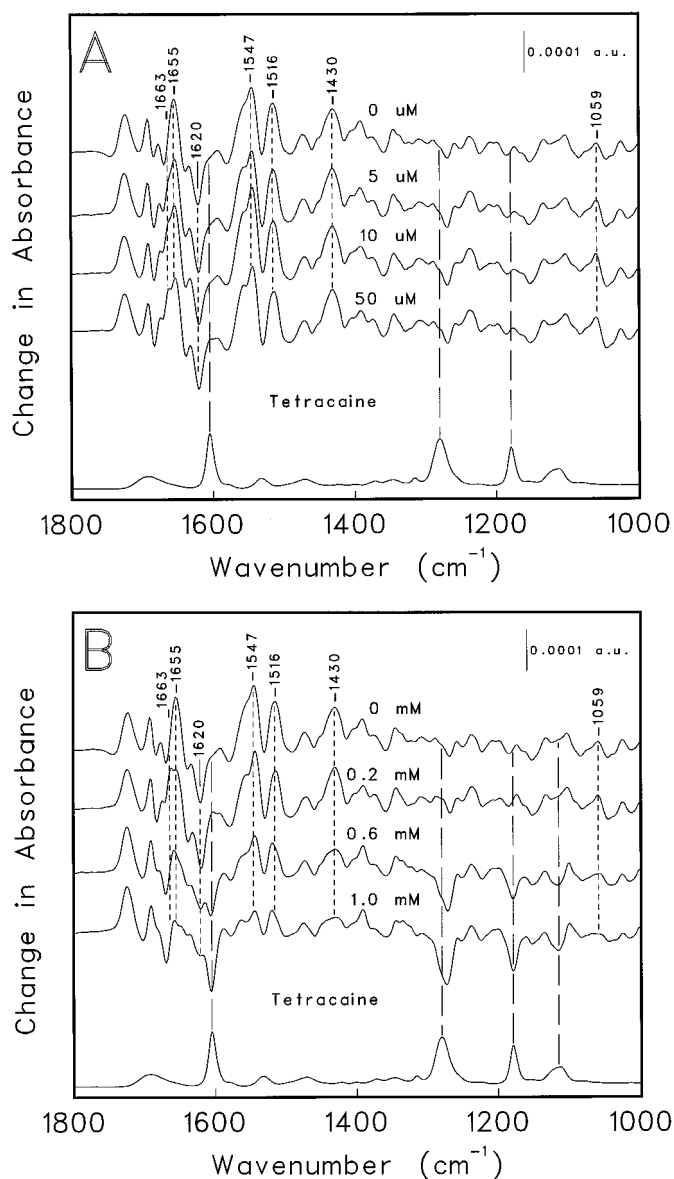


Fig. 3. Selected Carb difference spectra recorded in the presence of the noted concentrations of the LA tetracaine at concentrations consistent with its binding to either the NCB site within the ion channel pore (A) or both the neurotransmitter and NCB sites (B). Short, dashed lines identify spectral variations reflective of the resting-to-desensitized conformational change. Long, dashed lines identify negative bands reflecting the Carb-induced displacement of tetracaine from the nAChR. The bottom trace in both A and B is an absorbance spectrum recorded from a 50 μM tetracaine solution. The bar represents a change in intensity in the difference spectra of 0.0001 absorbance units (a.u.).

site lead to a marked loss of intensity near 1663, 1655, 1547, 1430, and 1059 cm^{-1} , suggestive of a shift back toward a desensitized nAChR (Figs. 3B and 4B). The decreases in intensity of all five bands occur with similar concentration dependencies, suggesting that the structural effects result from tetracaine action at a single class of sites. The spectra also exhibit negative bands indicative of the Carb-induced displacement of tetracaine from the neurotransmitter-binding site as well as a decrease in intensity of the two noted bands near 1620 and 1516 cm^{-1} , suggestive of the formation of direct physical interactions between tetracaine and neurotransmitter-binding site residues. These data show that desensitization can occur exclusively as a result of LA binding to the neurotransmitter-binding site. This finding is consistent with and explains the previously reported increase in acetylcholine-binding affinity that is observed at tetracaine concentrations higher than those necessary to saturate the NCB site (Boyd and Cohen, 1984). The conformational effects of tetracaine at the neurotransmitter-binding site overcome any changes in nAChR conformational equilibria that result from tetracaine binding to the NCB site.

Discussion

Previous studies have shown that the positive and negative bands that are observed in the difference between infrared spectra recorded in the presence and absence of Carb reflect structural changes that occur specifically upon the binding of Carb to the nAChR and subsequent desensitization (Baenziger et al., 1992, 1993). We show here that LAs elicit changes in the intensity of these positive and negative dif-

ference bands at concentrations consistent with the known binding affinities of LAs for the NCB- and/or neurotransmitter-binding sites (Table 1). The close correlation between the concentration dependencies of the spectral variations and the reported pharmacological properties of LAs at the nAChR provides compelling evidence that the detected spectral variations reflect changes in structure that result specifically from LA binding to the nAChR.

The Carb difference spectra show that dibucaine, prilocaine, lidocaine, and tetracaine all bind to the neurotransmitter site and that neurotransmitter-site binding leads to a conformational change in the nAChR. This conformational change is characterized by a loss of intensity in the difference spectrum at five frequencies centered near 1663, 1655, 1547, 1430, and 1059 cm^{-1} . The similarity of the spectral variations elicited by each LA suggests that each stabilizes a similar conformation of the nAChR by binding to the neurotransmitter-binding site. Because the LA-induced conformational change results from a mimicking of Carb binding to this site (see below), the loss of intensity at these five frequencies likely reflects the formation of a desensitized conformation equivalent to that stabilized by prolonged exposure to Carb. This interpretation is consistent with the observation of similar band intensity changes in Carb difference spectra recorded from the nAChR reconstituted into egg phosphatidylcholine membranes where the nAChR is not capable of undergoing agonist-induced desensitization (McCarthy and Moore, 1992; Ryan et al., 1996).

The Carb difference spectra also show that LA binding to the NCB site leads to conformational changes in the nAChR

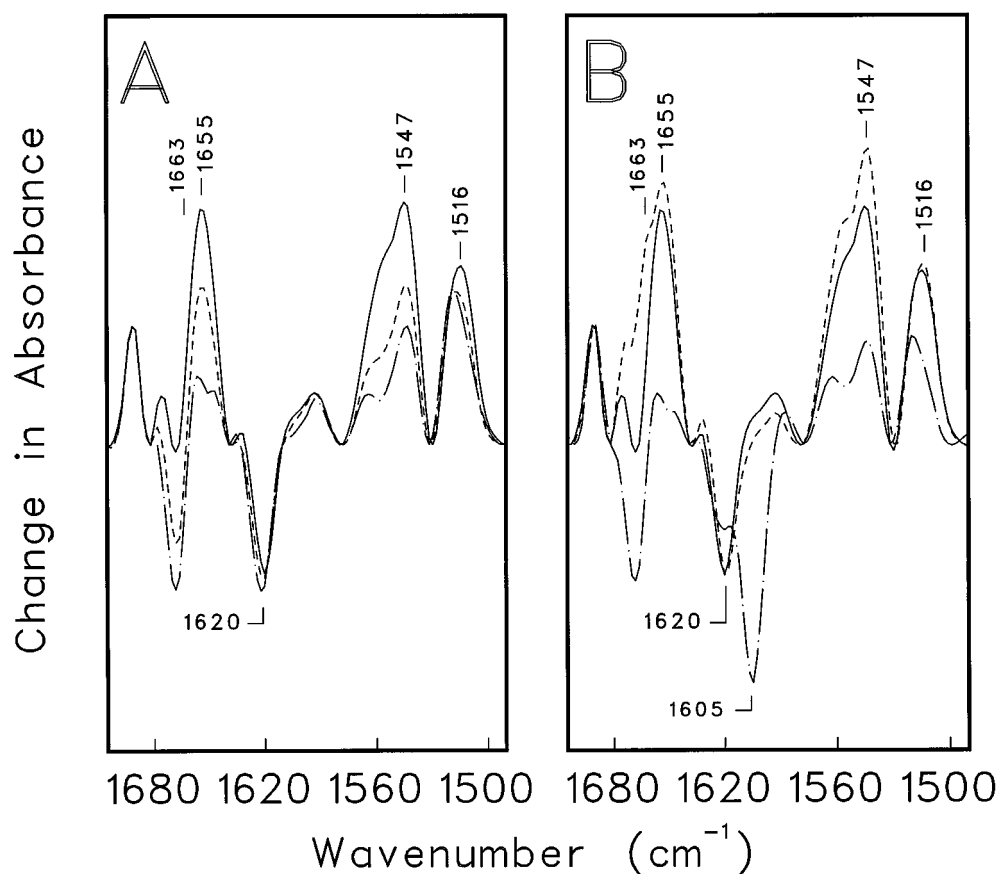


Fig. 4. Comparison of selected Carb difference spectra recorded in the absence (solid line in A and B) and presence of (A) 4 mM (---) and 10 mM (- · -) prilocaine and (B) 10 μM (---) and 1 mM (- · -) tetracaine. A multiple-point baseline correction was performed on all spectra to reduce small baseline fluctuations and thus allow for superimposition of spectra. Note that the baseline corrections do not affect the intensity or the frequency of the individual bands (compare spectra presented here with those presented in Figs. 2 and 3 that are not baseline-corrected). The negative band near 1605 cm^{-1} in B reflects the loss in tetracaine vibrational intensity due to the Carb-induced displacement of tetracaine from the neurotransmitter-binding site.

but that these changes are characterized mainly by variations in band intensity centered near 1663 and 1059 cm^{-1} . Prilocaine and lidocaine both lead to a loss of intensity in the difference spectra at 1663 and 1059 cm^{-1} . In contrast, tetracaine mainly leads to an increase in intensity of both difference bands. The opposing spectral effects of prilocaine/lidocaine and tetracaine are consistent with the opposing allosteric effects of these LAs on acetylcholine-binding affinity at the neurotransmitter-binding site. The binding of either prilocaine or lidocaine to the NCB site leads to an increase in binding affinity for acetylcholine whereas the binding of tetracaine leads to a decrease (Cohen et al., 1974; Boyd and Cohen, 1984). Significantly, because prilocaine, lidocaine, and tetracaine mainly influence the intensity of only two of the five difference bands that are indicative of agonist-induced desensitization, it can be concluded that LA binding to the NCB site leads to the formation of conformations that are structurally intermediate between the resting and desensitized states. These intermediate conformations have altered affinities for acetylcholine, even though they do not represent the formation of either a fully desensitized or sensitized nAChR.

The intermediate conformations of the nAChR stabilized by LA binding to the NCB site cannot yet be related to the various fast and slow desensitized states that have been identified previously using acetylcholine kinetic-binding studies (Weiland et al., 1977; Heidmann and Changeux, 1979a,b; Boyd and Cohen, 1980a,b). Regardless, it is clear that LAs stabilize multiple conformations of the nAChR by binding to the NCB and neurotransmitter sites. The conformation of the nAChR that is stabilized by a particular LA at a given concentration thus is dependent on the relative affinities of the LA for the neurotransmitter- and NCB-binding sites and either the complementary or competing conformational effects that result from LA binding to each of these two sites. Note that our data highlight the importance of neurotransmitter-site binding in terms of the mechanism of LA action at the nAChR. Tetracaine stabilizes a desensitized conformation by binding to the neurotransmitter site even though simultaneous binding to the NCB site favors the formation of a resting-like intermediate state. Desensitization can occur exclusively as a result of neurotransmitter-site binding and can dominate over conformational effects that result from binding to the NCB site.

In addition, some LAs such as chlorpromazine and trime-thisoquin bind to saturable low-affinity allosteric sites on the nAChR thought to be located near the lipid-protein interface (Heidmann et al., 1983). Although such low-affinity sites have not been demonstrated for dibucaine, prilocaine, and lidocaine, these LAs could bind to such low-affinity sites leading to some of the vibrational changes observed here in the difference spectra. However, despite large differences in the potencies of the three LAs, there is a close correlation between the concentrations of the LAs required to elicit the observed vibrational changes and the reported nAChR-binding affinities. This suggests that the structural changes result from interactions at the NCB- and neurotransmitter-binding sites. Consistent with this interpretation, all of the vibrational changes attributed to LA binding to the neurotransmitter site occur concomitant with the appearance of spectral features indicative of Carb-induced displacement of the LAs from the neurotransmitter site. The vibrational

changes attributed to a conformational change resulting from tetracaine binding to the NCB site occur at micromolar concentrations that are well below those expected for binding to low-affinity nAChR sites. Preliminary difference spectra recorded in the presence of the LA proadifen also exhibit spectral variations similar to those observed in the presence of dibucaine, lidocaine, and prilocaine, but at concentrations at which binding to the low-affinity sites should be minimal (unpublished observations). Although subtle structural effects of LA binding to low-affinity binding sites are possible, the observed spectral changes likely arise as a consequence of LA binding to either the NCB- or neurotransmitter-binding sites.

LA-induced changes in the Carb difference spectra suggest details regarding the nature of the physical interactions that occur between the LAs and nAChR-binding site residues. The relatively high concentrations of dibucaine, prilocaine, lidocaine, and tetracaine that result in binding to the neurotransmitter site lead to a reduction in the intensity of the two difference bands near 1620 and 1516 cm^{-1} . The vibration near 1516 cm^{-1} is highly characteristic of a tyrosine ring-stretching vibration. Several tyrosine residues are found in the neurotransmitter-binding site and perform a critical role in agonist binding likely via the formation of cation-tyrosine π electron interactions (Dennis et al., 1988; Dougherty and Stauffer, 1990; Cohen et al., 1991; Tomaselli et al., 1991; Aylwin and White, 1994; Sine et al., 1994). Carb-tyrosine interactions are critical for gating the nAChR ion channel. Carb difference spectra recorded in the absence of LAs using the Carb analog, tetramethylammonium, exhibit a 1516- cm^{-1} band of intensity comparable to that observed in Carb difference spectra (data not shown), indicating that the vibration is specific to interactions with the cationic ammonium group of Carb (unpublished observations). A substantial portion of the 1516- cm^{-1} intensity in Carb difference spectra recorded in the absence of a LA therefore is likely due to an increase in tyrosine vibrational intensity that results from the formation of cation- π electron interactions. The ability of some LAs to substantially reduce the intensity of this vibration upon binding to the neurotransmitter-binding site suggests that LAs mimic the cation-tyrosine interactions that perform a key role in agonist action at the nAChR.

Neither prilocaine nor lidocaine has as dramatic an effect on the intensity of the two vibrations near 1620 and 1516 cm^{-1} as do dibucaine and tetracaine. The binding of both prilocaine and lidocaine to the neurotransmitter-binding site is also much weaker than the binding of dibucaine and tetracaine (Table 1). Prilocaine and lidocaine differ structurally from the other two LAs in that they have only one instead of three atoms between the charged nitrogen and the carbonyl carbon (Fig. 1C). The reduced distance between the nitrogen and ester carbonyl could prevent binding to the neurotransmitter-binding site in a manner that allows both prilocaine and lidocaine to interact simultaneously at both the cationic and esterophilic agonist subsites (Michelson and Zeimal, 1973). Alternatively, the reduced number of carbon atoms also could bring the bulky aromatic groups of the LAs closer to the charged nitrogen and thus prevent tight binding of the LAs to the cationic-binding subsite. Although more detailed studies are required both to assign the two vibrations to specific amino acids and to interpret the LA-induced changes in intensity, the variable influence of the LAs likely reflect

subtle differences in how the LAs bind to the neurotransmitter site as a consequence of their slightly different chemical structures.

The binding of LAs to the neurotransmitter site and mimicry of agonist-induced desensitization likely is governed by the ammonium cation. All LAs that we have studied both possess a positively charged nitrogen moiety (Fig. 1C) and appear to stabilize the same conformation of the nAChR by forming interactions between the charged nitrogen- and neurotransmitter-binding site tyrosine residues. In contrast, LA action at the NCB site likely is governed by the hydrophobic substituents on the LAs. Acetylcholine and Carb, which lack large hydrophobic substituents, bind to the NCB site with extremely weak affinity whereas the interactions of neutral general anesthetics within the ion channel pore are governed by hydrophobicity (Forman et al., 1995). Note that a greater structural diversity is found in the hydrophobic moieties of the various LAs than is found with the charged nitrogen substituents (Fig. 1C). Structural differences in the hydrophobic substituents may be responsible for the contrasting conformational effects of lidocaine/prilocaine and tetracaine at the NCB site.

The ability to probe both the physical interactions that occur between LAs and amino acid side chains and the conformational states of the nAChR using a structure-based approach represents a step toward defining the molecular details of LA action at the nAChR. Further technical advancements should lead to kinetic infrared studies of acetylcholine binding to the various conformational states and thus allow us to relate the conformations defined here to those identified previously using acetylcholine kinetic-binding studies. The use of mutagenesis should also lead to the identification of those regions of the nAChR that are involved in the specific conformational changes and the specific residues that are involved in direct physical interactions with the bound LAs.

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