Binding Properties of Agonists and Antagonists to Distinct Allosteric States of the Nicotinic Acetylcholine Receptor Are Incompatible with a Concerted Model*

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Recent work has shown that the nicotinic acetylcholine receptor (nAChR) can be fixed in distinct conformations by chemical cross-linking with glutardialdehyde, which abolishes allosteric transitions in the protein. Here, two conformations that resemble the desensitized and the resting states were compared with respect to their affinities for different classes of ligands. The same ligands were tested for their ability to convert the nAChR from a conformation with low affinity to a conformation with high affinity for acetylcholine. As expected, agonists were found to bind with higher affinity to the desensitized state-like conformation and to induce a shift of the nAChR to this high affinity state. In contrast, although most antagonists tested bound preferentially to the desensitized receptor as well they failed to induce a change of the affinity for acetylcholine. These observations sharply contradict basic predictions of the concerted model, including the postulate of a preformed equilibrium between the different states of the nAChR in the absence of agonist. With a similar approach we could show that the non-competitive inhibitor ethidium is displaced in a non-allosteric manner by other well characterized channel blockers from the cross-linked nAChR. These results require revision of current models for the mechanisms underlying non-competitive antagonism at the nAChR.

The nicotinic acetylcholine receptor (nAChR)1 from the electric tissue of Torpedo californica is a prototype of the large family of ligand-gated ion channels (1, 2). It is an allosteric protein (3, 4) that can exist in at least three distinct, yet interconvertible conformational states (5): In the resting state the receptor has low affinity for acetylcholine and the ion channel is closed. Binding of two agonist molecules in a positively cooperative manner triggers gating of the intrinsic ion channel, which in turn leads to the permeation of cations. Prolonged agonist exposition induces receptor desensitization (6). In the desensitized conformation the nAChR has an increased affinity for acetylcholine as compared with the resting state, but the ion channel is closed. Each of these allosteric states is characterized by a distinct protein conformation.

The structure of the nAChR meets basic prerequisites of allosteric regulatory proteins: First, it is an oligomer formed by five subunits with the stoichiometry α2β2γδ. Second, given the homology of the subunits (7) and considering the fact that the neuronal α7-subunits can form functional homomeric receptors, one can assume that these subunits are arranged in a pseudo-symmetric manner. Indeed, in the pentamer α and the ω helical transmembrane segments M2 of each subunit contribute to a central ion channel (8), whereas the residual segments are oriented toward the plasma membrane or neighboring subunits. Third, the binding sites for agonists and competitive antagonists are located at the interfaces between neighboring subunits, i.e. in the case of Torpedo and of muscle-type nAChR an α-subunit and the adjacent γ- or δ-subunit (9–11). This emphasizes the importance of the quaternary structure for the cooperativity of ligand binding and receptor activity. Fourth, the active site of the protein, i.e. the ion channel, is located along the central axis of pseudo-symmetry.

Several studies demonstrated that agonist binding and channel gating occur at topographically distinct sites within the receptor molecule and further underlined the allosteric nature of channel activation. Among these are affinity labeling experiments in which an α-neurotoxin derivative formed a photo-induced cross-link with the upper part of the channel-forming M2 helix (12). According to this finding the distance between the binding sites for peptide antagonists and the ion channel is in the range of between 15 and 20 Å.

The lumen of the ion channel includes several rings of negatively charged amino acids contributed by all five M2 helices, which render the nAChR selective for cations. Many positively charged non-competitive antagonists (NCAs) exhibit high affinities to the nAChR (13) and bind to this luminar site when the receptor is in the open state. These NCAs are therefore described as channel blockers, which occlude the pore like a cork sticking in a bottle neck. Photoaffinity labeling experiments support this model, because some NCAs, among others such as triphenylmethylphosphonium (8, 14, 15), chlorpromazine (16–18), and tetracaine (19), have been shown to contact homologous residues within the channel-forming M2 helices. However, for some NCAs non-luminal binding sites have been postulated: Ethidium, for instance, binds in a mutually exclusive manner with other high affinity channel blockers (20), but the binding site is expected to lie in the outer vestibule of the ion channel, about 40 Å away from the entrance of the ion channel and slightly above the agonist binding sites (21). Quinacrine is believed to bind to a “peri-annular” locus (22), which is, in contrast to the ethidium locus, accessible to lipophilic and...
hydrophilic quenching molecules in the closed desensitized state (23) and in the open state (24). As a consequence of this view, the channel blocking properties of ethidium and quinacrine have been postulated to be mediated by allosteric mechanisms.

It has been demonstrated that allosteric transitions in the nAChR can be prevented by covalent cross-linking with homobifunctional reagents (25). The intermolecular connection of neighboring subunits reduces mobility at the interfaces and thereby apparently disturbs signal transfer between the agonist binding sites and the ion channel. Cross-linking in the absence of ligand “freezes” the receptor in a conformation with low affinity for acetylcholine that resembles the resting state, whereas cross-linking in the presence of carbachol yields a population of receptors in a high affinity conformation, which likely represents receptors in the desensitized state. Thus, it is now possible to examine the ligand binding properties of distinct conformations under well defined conditions, because the ligand itself cannot affect the conformation of the cross-linked nAChR.

In the present work, the two conformations resulting from cross-linking in the presence or absence of agonist were compared with respect to their affinities for agonists and competitive antagonists by competition experiments with $[^3H]$ACh. Furthermore, different classes of ligands were tested for their ability to convert the nAChR from the low affinity state to a conformation of high affinity for acetylcholine. Taken together the results of these experiments contradict basic predictions of the concerted (symmetry or MWC) model (26), including the postulate of a preformed equilibrium between the different receptor states in the absence of agonists. In addition, we monitored the fluorescence properties of the non-competitive antagonist ethidium in the two well-defined states after cross-linking and found that ethidium could be displaced by luminal NCAs even when allosteric transitions were abolished. The observations require revision of current models of the binding of nAChR channel blockers.

**EXPERIMENTAL PROCEDURES**

**Materials**—Liquid nitrogen-frozen tissue from Torpedo californica was supplied by C. Winkler (San Pedro, CA). Carbamoylcholine, ethidium bromide, eserine, ($\sim$)-nicotine, tetracaine, d-tubocurarine, and chlorpromazine were from Sigma (Deisenhofen, Germany). Triethylmethylphosphonium (TPMP$^+$) was from Aldrich (Steinheim, Germany), and glutardialdehyde was from Merck. $[^3H]$ACh and [alpha-3H]-bungarotoxin were purchased from NEN Life Science Products (Cologne, Germany).

**Preparation of nAChR-rich Membranes**—nAChR-rich membranes were prepared from the electric organ of T. californica as described previously (27). In the resulting suspension the concentration of nAChR was determined by [alpha-3H]-bungarotoxin binding assays (28).

**Cross-linking**—Cross-linking of nAChR-rich membranes with glutardialdehyde was performed in 50 mM Na$_2$H$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.4, with a final cross-linker concentration of 4 mM and a protein concentration of 1 mg/ml for 20–28 h at room temperature. Cross-linking in the presence of ligand was started after preincubation of nAChR-rich membranes with the respective ligand for 30 min at room temperature. After the cross-linking reaction, the ligand was removed from the mixture by repeated centrifugation (100,000 × g; 15 min) and thorough resuspension of the membrane pellet.

**Binding Assays**—Binding of $[^3H]$ACh (40 mCi/mmol) to nAChR-rich membranes was determined as described by Watty et al. (25). NTII from Naja naja oxiana was radioactively iodinated with $^{125}$I as described previously (29). As for $[^3H]$ACh binding assays, the centrifugation technique was used to determine binding of increasing concentrations of NTII to receptor-rich membranes in the native, the desensitized, and the resting state. The final nAChR concentration in the assay was 50 nM.

**Fluorescence Measurements**—All fluorescence measurements were performed at room temperature using an Amino Bowman spectrometer series 2 (Rochester, NY). Fluorescence of ethidium bound to the native or to defined allosteric states of the nAChR was determined by monitoring emission at 595 nm upon excitation at 490 nm. As for all following fluorescence measurements the slit widths were 4 nm for both excitation and emission. To obtain the concentration of specifically bound ethidium binding, curves had to be corrected for the unspecific component of ethidium fluorescence. Therefore, in a parallel cuvette, ethidium fluorescence was monitored under the same conditions in the presence of the competitive antagonists gallamine (50 μM; left panel) and hexamethonium (100 μM; right panel); and in the presence of the non-competitive antagonists ethidium (10 μM; left panel) and TPMP$^+$ (50 μM; right panel). Binding data are shown in Scatchard plots. In each experiment, control curves of nAChR-rich membranes fixed in the presence of 100 μM carbamoylcholine (solid squares) are shown for determining the maximal number of binding sites.

**RESULTS**

**A.** Agonists Exclusively Convert the nAChR from a Conformation with Low Affinity to a Conformation with High Affinity for Acetylcholine—It was shown previously by Watty et al. (25) that the nAChR can be fixed in distinct conformational states by covalent cross-linking with different homobifunctional reagents. Accordingly, in our experiments, treatment of nAChR-rich membranes in the absence of agonist with the non-desensitizing cross-linker glutardialdehyde resulted in a population of low affinity binding sites ($K_d = 0.7 ± 0.2$ μM) for acetylcholine (Fig. 1A, left panel), whereas after cross-linking in the presence of carbachol binding sites with high affinity for ace-

![FIG. 1 Influence of various types of ligands on the conformational state of the nAChR](image-url)
tylcholine ($K_a = 26.5 \pm 3.5 \text{ nM}$) were obtained. These two different receptor conformations have been interpreted to be the resting and the desensitized state, respectively. As indicated by the linearity of the Scatchard plot and a Hill coefficient of 1, cooperativity of agonist binding was lost after glutardialdehyde treatment. In the conformation resembling the resting state, a minor population (25–35%) of high affinity binding sites was observed, which was explained by a preformed equilibrium existing between the different states of the nAChR in the absence of ligand. To test the validity of this explanation, nAChR-rich membranes were preincubated with various ligands. After the cross-linking procedure, the tested ligands were removed by repeated centrifugation. Finally, the affinity state of the resulting membrane preparation was monitored by $[^3H]$ACh binding assays. According to the concerted model, a change of the proportion of high affinity binding sites as compared with the resting state-like conformation is expected for those ligands that show preferential binding to one of the two states.

As in the case of carbachol, cross-linking in the presence of (−)-nicotine yielded a homogenous population of high affinity binding sites (Fig. 1A, right panel). This observation clearly demonstrates that the presence of agonist during cross-linking triggers a conversion of the nAChR from a conformation of low affinity to a conformation of high affinity for acetylcholine. In contrast to agonists, the competitive antagonists hexamethonium, d-tubocurarine, or gallamine failed to induce a change of the affinity status of the agonist binding sites (Fig. 1B). The nAChR remained in a conformation with low affinity for acetylcholine. A subpopulation of high affinity sites was detected; however, the size of this population was not altered (35%, 33%, and 32% for hexamethonium, d-tubocurarine, and gallamine, respectively) as compared with the preparation obtained by cross-linking in the absence of ligand. A similar result was obtained when the non-competitive antagonists ethidium and TPMP$^+$ (30% and 34% high affinity binding sites, respectively) were tested for their capability to affect the conformation of the nAChR in the absence of agonist (Fig. 1C). Thus, apparently exclusively, agonists had the potency to convert the nAChR from the resting state-like to the desensitized state-like conformation. Upon exposition to all antagonists the receptor remained in the resting state-like conformation.

**Affinity of Agonists and Competitive Antagonists for Distinct Receptor Conformations**—An important prediction of the concerted model for nAChR activation is that agonists act by shifting the preformed equilibrium toward the open state, whereas antagonists exert their inhibiting effect by stabilizing the resting state. Both predictions would be consistent with the findings described above as long as antagonists had higher affinity for the nAChR in the resting state. Therefore, in a second approach we cross-linked native nAChR-rich membranes in the absence or presence of carbachol. Then competition studies with 2 μM $[^3H]$ACh were performed with the membrane preparations to evaluate the affinity of different ligands for the two preparations. As expected all tested agonists preferentially bound to the nAChR cross-linked in the presence of agonist (Table I) with the highest affinity observed for acetylcholine as compared with carbamoylcholine (0.5 μM in the desensitized state-like versus 35 μM in the resting state) or (−)-nicotine (1.4 versus 120 μM). The antagonist gallamine bound dramatically stronger to the desensitized state-like conformation as well (1.9 μM versus 1 μM). Hexamethonium, on the other hand, had similar affinity for both preparations (350–550 μM).

Likewise, the larger peptide antagonists α-neurotoxin II from *N. naja oxiana* (NTII) was not able to discriminate between the two conformations (Fig. 2): Scatchard plots of $[^25]$I]NTII binding reveal that α-neurotoxins bind with similar affinities ($K_a = 16 \pm 3 \text{ nM}$) to receptors cross-linked in presence or absence of agonist. The dissociation constants for NTII binding to the cross-linked nAChR are about three times lower than the one for NTII binding to untreated nAChR-rich membranes (5 nM; data not shown).

**Binding of the Non-competitive Antagonist Ethidium to Distinct Receptor Conformations**—The NCA ethidium has been shown to be a potent blocker of the nAChR (31). Binding of ethidium is accompanied by an enhancement of fluorescence intensity and a blue shift in the emission maximum as compared with ethidium dissolved in buffer (20). Like many other NCAs, ethidium binds preferentially to the nAChR in the presence of agonist. Here we used the fluorescence of ethidium to examine binding properties of distinct receptor conformations for NCAs in the absence of other ligands. As can be seen in Fig. 3A, TPMP$^+/\text{tetracaine-sensitive fluorescence excitation and emission maxima of ethidium bound to the two cross-linked conformations were similar to those of ethidium bound to native nAChR-rich membranes. All three fluorescence spectra showed an excitation maximum at 505 nm and an emission maximum at 587 nm. However, differences were visible with regard to fluorescence intensity: The fluorescence of ethidium bound to nAChR-rich membranes cross-linked in the absence of agonist was rather weak. Importantly, in contrast to native receptor-rich membranes, the fluorescence intensity was not significantly enhanced upon addition of carbamoylcholine. This proves that the receptor could not be converted to a conformation of high affinity for ethidium after being locked in the resting state-like conformation. The fluorescence of ethidium bound to nAChR-rich membranes cross-linked in the presence of agonist was much stronger; it was not affected by the addition of agonist as well. As compared with native receptor-rich membranes in the presence of desensitizing concentrations of carbamoylcholine we observed an approximate 30% loss in fluorescence intensity, indicating a direct influence of lysine modification by glutardialdehyde on ethidium binding. This loss was even stronger when higher concentrations of glutardialdehyde were applied during the cross-linking procedure (data not shown).

Fluorescence titrations (Fig. 3B) were performed to determine the affinity of ethidium for native receptor-rich membranes as well as for the two cross-linked membrane preparations. We found that fluorescence of ethidium bound to cross-linked nAChR-rich membranes was sensitive to the presence of non-competitive inhibitors (see below and Fig. 4). Thus, the resulting titration curves were corrected for unspecifically

| Ligand                  | Desensitized state-like conformation | Resting state-like conformation |
|------------------------|-------------------------------------|---------------------------------|
| Acetylcholine          | 0.026 ± 0.004                       | 0.7 ± 0.2                       |
| Carbamoylcholine       | 0.522 ± 0.070                       | 35 ± 10                         |
| (−)-Nicotine           | 1.36 ± 0.18                         | 120 ± 25                        |
| Gallamine              | 1.86 ± 0.25                         | 1040 ± 220                      |
| Hexamethonium          | 336 ± 45                            | 550 ± 120                       |
| Ethidium$^*$           | 0.25 ± 0.05                         | 2 ± 0.12                        |
| NTII$^*$               | 0.016 ± 0.005                       | 0.016 ± 0.002                   |

*As determined by fluorescence titrations.

$^*$As determined by $[^25]$I]NTII binding.

### Table I

Affinities of various ligands for distinct conformations of the nAChR

Apparent dissociation constants ($K_a$) were determined by competition experiments with 2 μM $[^3H]$ACh after fixing nAChR-rich membranes in the resting state-like or in the desensitized state-like conformation by covalent cross-linking with glutardialdehyde. $K_a$ values are given in μM. Please note that the determination of $K_a$ values is based on different ACh affinities in the two states.
bound ethidium, which was quantified by measuring fluorescence in the presence of saturating concentrations of tetracaine/TPMP⁺. Scatchard plots revealed that in each case ethidium was bound to a single class of binding sites. The affinity of ethidium was larger for native nAChR-rich membranes \( (K_D = 75 \text{ nM}) \) than for receptors cross-linked in the presence of agonist \( (K_D = 250 \text{ nM}) \). The dissociation constant of ethidium binding to the nAChR in the resting state-like conformation was in the range of 2 \( \mu \text{M} \). This demonstrates that ethidium does not only bind to the nAChR in the open but also in the closed desensitized or resting conformations, yet with different affinities.

**Luminal Binding Site of Ethidium**—Because the localization of the ethidium binding site is still a matter of debate, we addressed this question with our approach: It was proposed earlier that ethidium bound to the upper part within the receptor funnel, slightly above the agonist binding sites (21). According to this localization, the inhibiting effect would be mediated by an allosteric mechanism, because the binding event affected the function of the ion channel about 30 Å away. Therefore, we performed competition experiments with other NCAs, namely TPMP⁺ (14), chlorpromazine (16–18), and tetracaine (19), all of which have been proven to bind to the channel lumen by photoaffinity labeling. As shown in Fig. 4A increasing concentrations of these compounds induced a continuous reduction of the emission of ethidium bound to nAChR-rich membranes that had been induced in the desensitized state-like conformation in the presence of carbamoylcholine. This reduction was not due to quenching mechanisms as determined by measuring ethidium fluorescence in buffer with increasing concentrations of NCA (data not shown). The apparent dissociation constants determined for the three NCIs from the competition experiments performed with locked receptors are close to the values obtained for native nAChR-rich membranes. Because cross-linking has been shown to abolish allosteric mechanisms, we concluded that the compounds tested competed directly for the ethidium binding site. This is supported by the notion that in all cases Hill coefficients were close to 1 (Table II), indicating that ethidium was displaced from a homogenous population of binding sites in a non-allosteric manner.

**DISCUSSION**

**Ligand Binding to Distinct Conformations of the nAChR after Covalent Cross-linking**—In our study ligand binding properties of defined receptor conformations were correlated with respective predictions of the MWC model, which was established to explain the allosteric mechanism of channel activation in the nAChR. According to this model a preformed equilibrium should exist between the different states of the nAChR even in the absence of agonist (4); this equilibrium should be shifted by the addition of a given ligand to the state that has higher affinity for the respective compound. We found that, exclusively, agonists induced receptor desensitization. Consistent with previous observations (25) the nAChR remained in the resting state when the cross-linking was done in the absence of agonist. When competitive or non-competitive antagonists were applied during the cross-linking procedure, the receptor remained in a resting state-like conformation as deduced from the low affinity for \(^{3} \text{H} \text{ACh} \). The affinity of neurotoxin II from *N. naja oxiana* in the resting and the desensitized state. nAChR-rich membranes have been cross-linked in the presence or absence of 100 \( \mu \text{M} \) carbamoylcholine prior to binding assays with \(^{125} \text{I} \text{NTII} \). As shown in Scatchard analysis, the affinity is similar in the resting state-like (solid line) and the desensitized state-like (dotted line) conformations.

**FIG. 2. Affinity of neurotoxin II from N. naja oxiana in the resting and the desensitized state.** nAChR-rich membranes have been cross-linked in the presence or absence of 100 \( \mu \text{M} \) carbamoylcholine prior to binding assays with \(^{125} \text{I} \text{NTII} \). As shown in Scatchard analysis, the affinity is similar in the resting state-like (solid line) and the desensitized state-like (dotted line) conformations.
The nAChR.

of the dissociation curves into Hill-like plots. Slopes of one indicated
caine (595 nm upon excitation at 490 nm. Ethidium was displaced by tetra-
mation resembling the desensitized state. Emission was monitored at
desensitized and the resting state. Alternatively, the sequen-
cause it includes agonist-independent conversions between the
model, however, is not supported by our results as well, be-
agonist binding sites can act independently. This uncoupled
properties of epibatidine and acetylcholine to muscle nAChR;
that a concerted model can not accurately describe the binding
nAChR in the resulting conformation, this shift should be de-
shift this equilibrium toward the desensitized state. Because
bind preferentially to the desensitized conformation, should
TABLE II
Equilibrium dissociation constants for NCA binding to nAChR-rich
membranes fixed in the desensitized state-like conformation
Affinities were determined by competition experiments with 6.6 μM
ethidium. 1 nm carbamoylcholine was added to prevent NCA binding to
the acetylcholine binding sites. K_{app} values are given in μM. n_{H} = Hill
coefficient.

| Ligand       | n_{H}    | K_{app}    |
|--------------|----------|------------|
| Ethidium     | 1.02 ± 0.07 | 0.25 ± 0.06 |
| TPMP^+       | 0.92 ± 0.04 | 0.32 ± 0.11 |
| Chlorpromazine| 1.21 ± 0.15 | 0.53 ± 0.13 |
| Tetracaine   | 1.23 ± 0.08 | 6.38 ± 0.42 |

desensitized state-like conformation (Table I). It is also known that the
non-competitive antagonist TPMP^+ can be cross-linked to the δ-subunit much more efficiently in the desensitized state than in
the resting state (14, 15). In addition we could show by
fluorescence titrations that ethidium binds with almost 10-fold
higher affinity to the desensitized nAChR than to the resting
nAChR (Fig. 3B).

These observations sharply contradict the prediction that a
preformed equilibrium exists between the different allosteric
states of the nAChR (26): According to the MWC model
ethidium, TPMP^+ and gallamine, which have been shown to
bind preferentially to the desensitized conformation, should
shift this equilibrium toward the desensitized state. Because
this shift is supposed to be accompanied by an overall confor-
mational change of the protein, it should also affect the struc-
ture of the agonist binding sites. Thus, after “freezing” the
nAChR in the resulting conformation, this shift should be de-
tectable by an increase of the affinity of the binding sites for
acetylcholine. However, this was not the case.

In a different approach, Prince and Sine (32) demonstrated
that a concerted model can not accurately describe the binding
properties of epibatidine and acetylcholine to muscle nAChR;
they proposed an uncoupled model according to which the two
agonist binding sites can act independently. This uncoupled
model, however, is not supported by our results as well, be-
cause it includes agonist-independent conversions between the
desensitized and the resting state. Alternatively, the sequen-
tial model (33) suggests that the binding of the agonist (and not
an antagonist) molecule triggers a specific conformational
change at the subunit interfaces, which propagates to the chan-
nel lumen and finally leads to receptor activation and subse-
quent desensitization. In support of this model, multiple sub-
conductance states have been observed for the nAChR (34, 35)
indicating that the two α-subunits can act independently.

The observation that spontaneous openings of the nAChR
occur even in the absence of ligand (36) has been interpreted in
support of the MWC model. Whenever we detected the low
affinity conformation of the nAChR after covalent cross-link-
ing, a significant proportion (about 25–35% of B_m) of high
affinity binding sites was detected in parallel. This proportion
could not be reduced by the prolongation of the cross-linking
procedure or by an increase of the concentration of glutardial-
dehyde used (25). Thus, its existence is not due to incompletely
fixed nAChR-rich membranes. Considering that spontaneous
opening events are rare and brief (36) and have properties that
differ from those of agonist-induced openings (37), we believe
that the population of high affinity binding sites observed here
is too big to be indicative of a hypothetic equilibrium existing
between different states. More likely, these high affinity sites
are an artifact of the preparation representing simply non-
functional receptor molecules. This is mirrored by an approxi-
mate 30% reduction of the number of binding sites for ethidium
upon cross-linking (see below).

It was shown for mouse muscle nAChR (38, 39) that the
mutation of a highly conserved leucine residue in the middle of
the M2 domain (Leu-251 in the α-subunit) causes significant
shifts of the dose-response curves to lower acetylcholine con-
centrations. On first glimpse this observation seems to be an-
other argument in favor of the MWC model, because it indi-
cates that residues in the pore region influence the structure of
the agonist binding sites. However, these shifts were not based
on a higher affinity of the ligand but by a change of the chan-
nel’s open time. Therefore, in muscle-type nAChR each residue
in the leucine ring contributes to the destabilization of the
arrangement of the five M2 helices in the channel’s open state;
the mutation does not affect the structural determinants of the
agonist binding site. Similarly, the effects of the mutations
εT264P or βT265P within a ring of conserved serine/threonine
residues are restricted to the ion channel (40). In the δ-subunit,
however, the mutation S268P influences in a yet unknown way
channel activation as well as agonist binding.

In neuronal homomeric α7-receptors (41), the role of the
conserved leucine residue is apparently more complex. The
mutation L247T does not only alter the EC_{50} but also produces
an additional conductance state and converts the competitive
agonists hexamethonium and dihydro-β-erythroidin of the
wild type receptor into agonists (42). The structural basis for
these pleiotropic changes is still unclear, and it is possible that
allosteric transitions in α7-receptors have properties that are
different from those in muscle nAChR.

In contrast to smaller antagonists, snake peptide toxins were
found to be unable to discriminate between the desensitized
and the resting states. We conclude that α-neurotoxins do not
act by preferentially stabilizing the resting conformation of
the nAChR as proposed earlier (43) but inhibit agonist binding in a
direct competition for overlapping binding sites. Interestingly,
we observed a slight but significant reduction of the toxin’s
affinity after cross-linking native nAChR-rich membranes. This
might indicate an influence of mobility at the subunit
interfaces on the access of α-neurotoxins as discussed recently
(29). The relatively low affinity observed in our study for
gallamine in the resting state-like conformation might have simi-
lar reasons.

Luminal Localization of the Ethidium Binding Locus—In the
second part of our study we examined the interaction of the fluorescent NCA ethidium with defined conformations of the nAChR. Our results demonstrate that ethidium has similar fluorescence properties with respect to emission and excitation maxima, when bound to desensitized nAChRs before and after cross-linking, and therefore, is exposed to similar environments. However, the approximate 4-fold reduction of the affinity of ethidium for the nAChR after cross-linking indicates that glutaraldehyde treatment significantly affects the binding site.

Three well-characterized luminal NCAs could displace ethidium from its binding site even though allosteric transitions have been abolished by covalent cross-linking (Fig. 4). This strongly suggests that ethidium is displaced by a direct competitive mechanism from an overlapping luminal binding site and not by wide-range allosteric effects. Taken together with the observation that quinacrine directly competes with ethidium for a common locus on the nAChR (44), our observations suggest that all high affinity NCAs bind in a mutually exclusive manner to the luminal of the nAChR. The geometry of the channel lumen is affected by changes of the conformational state of the receptor so that different NCAs show different preferences for the one or the other state. However, the finding that tetracaine and TPMP+ or chlorpromazine contact the same residues in the resting and the desensitized state, respectively, implies that the M2 helices differ only slightly in their structure in the two orientations. Therefore, the structural rearrangements accompanying receptor desensitization are less pronounced than expected (45).

Because ethidium fluorescence increases upon binding and cannot be quenched efficiently by D2O or iodide (46), its high affinity binding site is most probably located in a hydrophobic environment (50). Furthermore, the low association and dissociation rate constants for tetracaine in the resting state were interpreted such that access to the tetraaine binding site is hindered and apparently depends on slow structural rearrangements in the channel region (19).

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