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Fixation of allosteric states of the nicotinic acetylcholine receptor by chemical cross-linking

(allosterism/desensitization/equilibrium binding)

ANKE WATTY*, CHRISTOPH METHFESSEL†, AND FERDINAND HUCHO*‡

*Institut für Biochemie, Fachbereich Chemie, Freie Universität Berlin, Thielallee 63, D-14195 Berlin, Germany; and †Zentrale Forschung, Abteilung Biophysik, Bayer AG, D-51368 Leverkusen, Germany

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ABSTRACT **Receptor activity can be described in terms of ligand-induced transitions between functional states. The nicotinic acetylcholine receptor (nAChR), a prototypic ligand-gated ion channel, is an "unconventional allosteric protein" which exists in at least three interconvertible conformations, referred to as resting (low agonist affinity, closed channel), activated (open channel), and desensitized (high agonist affinity, closed channel). Here we show that 3,3′-dimethyl suberimidate (DMS) is an agonistic bifunctional cross-linking reagent, which irreversibly "freezes" the nAChR in a high agonist affinity/closed-channel state. The monofunctional homologue methyl acetoimidate, which is also a weak cholinergic agonist, has no such irreversible effect. Glutardialdehyde, a cross-linker that is not a cholinergic effector, fixes the receptor in a low-affinity state in the absence of carbamoylcholine, but, like DMS, in a high-affinity state in its presence. Covalent cross-linking thus allows us to arrest the nAChR in defined conformational states.**

The nicotinic acetylcholine receptor (nAChR) is a heteropentameric transmembrane glycoprotein with the subunit stoichiometry $\alpha_2\beta\gamma\delta$. The five subunits are arranged around a central pore, which is permeable for cations in the receptor's activated state (1–3).

The nAChR is an allosteric protein (4, 5) existing in different interconvertible states (4, 6). In the absence of an agonist the receptor stays in a resting or closed channel state. After binding of two agonist molecules in a positive cooperative manner, the receptor is activated and the channel opens. During longer agonist exposure the receptor desensitizes, which means that the receptor closes while acetylcholine (ACh) is still bound (7).

Although the nature of allosteric transitions in oligomeric proteins is still under debate (8, 9) there is agreement that distinct allosteric states are characterized by distinct protein conformations. Because allosteric proteins are oligomeric proteins, conformational changes must be transduced from the ligand-binding sites to neighboring subunits (10). That such changes in quaternary structure accompany allosteric transitions has been shown at the atomic level by x-ray crystallography for many proteins—e.g., hemoglobin (11) and aspartate carbamoyltransferase (12, 13). These examples show that the subunit interfaces play a pivotal role in the interconversion of different allosteric states, which are characterized by different quaternary structures.

For the nAChR it has been established that the two binding sites for agonists and competitive antagonists are each located at the interface between an α-subunit and the neighboring γ- or δ-subunit (14–18). Therefore one can assume that the contact sites between subunits play an important role in the function and allosteric properties for the nAChR as well.

To date we know very little about the structural changes going on upon receptor activation and desensitization. Unwin and co-workers (19) examined the structure of the nAChR in the absence and presence of desensitizing amounts of carbamoylcholine by using cryoelectron microscopy of two-dimensional tubular crystals. Their work gave hints that changes in the quaternary structure may occur upon desensitization, especially affecting the γ- and δ-subunits. In x-ray crystallographic studies with hemoglobin it has been demonstrated that covalent cross-linking of neighboring subunits is a powerful method for fixing distinct conformational states (20) because such cross-linking abolishes mobility at the subunit interfaces. The homobifunctional lysine-specific cross-linking reagent 3,3′-dimethyl suberimidate (DMS) has been shown to cross-link neighboring subunits in Triton-solubilized nAChR from *Electrophorus electricus* (21) and *Torpedo californica* (22) to give rise to subunit dimers, trimers, tetramers, and pentamers. Cross-linking the subunits in the receptor pentamer to each other should lead to a more rigid structure and affect allosteric transitions.

In the present work, the effects of cross-linking on the allosteric and functional properties of the nAChR were studied with [^{3}H]ACh binding on nAChR-rich membranes from *Torpedo californica* and with electrophysiological current recordings from *Xenopus* oocytes expressing the nAChR from embryonic rat muscle (the structures of the cross-linking reagents used are given in Fig. 1).

MATERIALS AND METHODS

Preparation, Solubilization, and Reduction of the nAChR from *Torpedo californica*. nAChR was prepared from the electric organ of *Torpedo californica* (Winkler Enterprises, San Pedro, CA) as described (23).

Solubilization of the nAChR was done by incubation with Triton X-100 (Calbiochem) in a final concentration of 0.1% for 30 min at room temperature. The sample was centrifuged at 100,000 × *g* for 15 min; the supernatant contains the solubilized nAChR. For reduction of the δ-δ disulfide bond, 2-mercaptoethanol (Sigma) was added to a final concentration of 1% (vol/vol), and the mixture was incubated for 1 h at room temperature.

Cross-Linking. Cross-linking of nAChR-rich membranes with DMS and DTBP (Pierce) was performed in 0.2 M triethanolamine, pH 8.5, for 1 h at room temperature with a final concentration of 2 mg/ml (protein concentration 1 mg/ml). Triton-solubilized receptor was cross-linked at 1 mg/ml for 1 h at room temperature (protein concentration 0.5 mg/ml).

Cross-linking with BS^3 (Pierce) was done in 20 mM phosphate buffer, pH 7.4, as with DMS. Cross-linking with glutardialdehyde

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Abbreviations: nAChR, nicotinic acetylcholine receptor; DMS, 3,3′-dimethyl suberimidate; MAI, methyl acetoimidate; ACh, acetylcholine; BS^3, bis(sulfosuccinimidyl) suberate; DTBP, dimethyl 3,3′-dithiobispropionimidate.

‡To whom reprint requests should be addressed at: Freie Universität Berlin, Fachbereich Chemie, Institut für Biochemie, Thielallee 63, D-14195 Berlin, Germany. e-mail: hucho@chemie.fu-berlin.de.

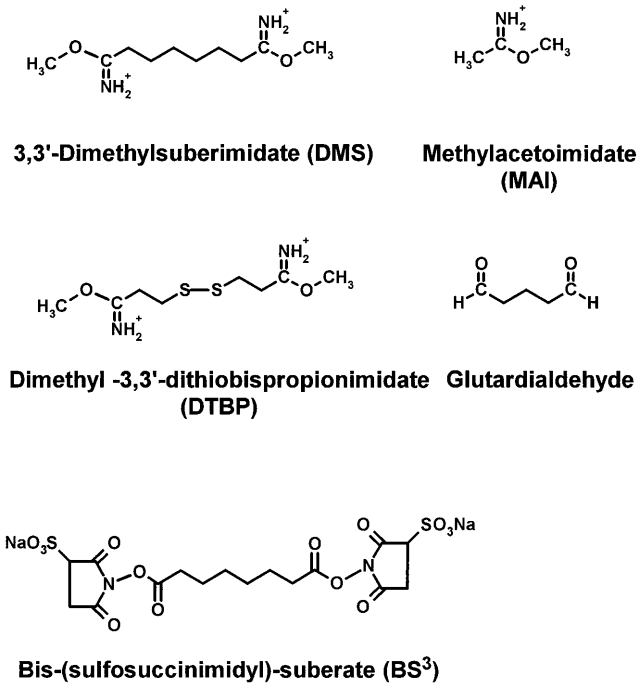

FIG. 1. Structures of the cross-linking reagents used in this work.

(Merck) was carried out in 20 mM phosphate buffer, pH 7.4, with a final concentration of 0.04% (4 mM) for 3–20 h at room temperature. As with DMS, cross-linking of nAChR-rich membranes with glutardialdehyde was done with a protein concentration of 1 mg/ml, and cross-linking of Triton-solubilized nAChR was carried out with a protein concentration of 0.5 mg/ml. Cross-linking with glutardialdehyde in the presence of carbamoylcholine was done by preincubating with 0.1 mM carbamoylcholine for 10 min at room temperature. After cross-linking, carbamoylcholine was removed by repeated centrifugation (100,000 × *g*, 10 min) and resuspension of the pellet.

Modification with methyl acetoimidate (MAI) was done as with DMS. MAI was synthesized as described (24). All reactions were stopped with an excess of Tris·HCl at pH 7.4.

Cross-linking of rat muscle nAChR expressed in *Xenopus* oocytes was done in frog Ringer's solution (115 mM NaCl/2.5 mM KCl/1.8 mM $CaCl_2$/10 mM Hepes, pH 7.2) with 2 mg/ml DMS.

Gel Electrophoresis of Cross-Linked nAChR. Gel electrophoretic separation was done as described (25) using 4–5% polyacrylamide rod gels (see figure legends). For each gel, 50 μg of protein was applied.

[^{3}H]ACh Binding Assay. nAChR in Ringer's solution (160 mM NaCl/5 mM KCl/2 mM $MgCl_2$/2 mM $CaCl_2$/3 mM Na_xPO_4, pH 7.0) was incubated with 0.1 mM eserine (Sigma) for 30 min at room temperature to block acetylcholinesterase. Increasing concentrations of [^{3}H]ACh (75 mCi/mmol; DuPont/NEN; 1 mCi = 37 MBq) were added to a constant amount of protein (32–130 μg depending on the K_d) and incubated for 30 min at room temperature. The total volume per sample was 200 μl. The samples were centrifuged for 10 min at 80,000 × *g* in a Beckmann tabletop ultracentrifuge at 20°C. Aliquots were taken before and after centrifugation to obtain the concentration of total and free [^{3}H]ACh, respectively.

For competition experiments nAChR was incubated with 1.7×10^{-7} M [^{3}H]ACh for 30 min at room temperature, and increasing concentrations of effector (DMS or MAI) were added and centrifuged without any further incubation to minimize hydrolysis and covalent reaction of the effector.

Electrophysiology. Culture and electrophysiological measurements of oocytes from *Xenopus laevis* have been described in detail elsewhere (26). The oocytes were injected with 20–50 nl of cDNA solution (10 ng/μl), containing the expression vector with the rat muscle nAChR clones α, β, γ, and δ in the ratio 2:1:1:1. The cDNA was kindly provided by V. Witzemann (Max-Planck-Institut für Medizinische Forschung, Heidelberg).

Whole-Cell Current Measurements. Whole-cell current measurements were mainly done as described (26). Microelectrodes were pulled and filled with 1.5 M potassium acetate/100 mM KCl. The pipette resistance was less than 0.5 MΩ. The oocytes were voltage-clamped at −80 mV using a Tec cc-02 amplifier (upi, electronic, Tamm). Substances were applied by perfusion (3–10 ml/min) in frog Ringer's solution using electrically controlled valves. Data were recorded using a Tektronic 2221A digital storage oscilloscope and transferred to a personal computer for analysis.

RESULTS

Cross-Linking of the nAChR with DMS Enhances the [^{3}H]ACh Affinity with a Loss of Cooperativity. Treating Triton-solubilized nAChR with low DMS concentrations mainly causes intramolecular cross-linking; intermediate stages of cross-linking (subunit dimers, etc.) can be seen (Fig. 2*A*, lane 2). This proves the existence of intersubunit cross-links. In contrast, cross-linking of native nAChR in *Torpedo* membrane vesicles with a high receptor density, using a high DMS concentration, leads to intra- and intermolecular cross-linking, which results in receptor polymerization (Fig. 2*A*, lane 3).

For [^{3}H]ACh binding experiments nAChR-rich membranes were cross-linked with a high DMS concentration to get a complete cross-linking of the whole receptor pentamer.

Native nAChR from *Torpedo californica* binds [^{3}H]ACh with positive cooperativity (Hill coefficient $n_H = 1.6 \pm 0.2$) and high affinity ($K_{0.5} \approx 75 \pm 5$ nM). This is characteristic for the high-affinity desensitized state occurring upon equilibrium binding after long [^{3}H]ACh exposure (Fig. 2*B*). Receptor cross-linked with DMS shows a slightly higher affinity for [^{3}H]ACh ($K_d \approx 50 \pm 5$ nM) with a loss of cooperativity ($n_H = 1 \pm 0.2$, Fig. 2*B Inset*; see also Table 1 for a list of the binding constants). This result implies that, as a consequence of DMS cross-linking, the receptor already existed in the high-affinity state before it was exposed to [^{3}H]ACh in the binding assay. Maximal [^{3}H]ACh binding, however, is unaltered, indicating that cross-linking does not take place within the binding site, nor does it sterically affect ligand binding.

Cross-Linking with DMS Abolishes ACh-Evoked Currents at Rat Muscle nAChR Expressed in *Xenopus* Oocytes. The state of functional activity of the ion channel after cross-linking was studied with whole-cell current measurements on rat muscle nAChR expressed in *Xenopus* oocytes. DMS cross-linking results in a profound reduction of the inward current amplitude induced by ACh to less than 10% after 30 min of cross-linking (Fig. 3*A*) and to no current at all after 1 h. The current response cannot be recovered by extensive washing and is therefore presumably due to the covalent cross-linking. After 30 min of cross-linking, the remaining activatable receptors show a normal ACh dose–response curve with a Hill coefficient of $n_H = 1.6 \pm 0.2$ (Fig. 3*B*). We therefore conclude that cross-linked receptors are irreversibly fixed in a nonconducting state and that the cross-linking reaction is complete after 1 h.

DMS and Other Imidates Are Cholinergic Agonists. An interesting question is, why does DMS fix the nAChR in its desensitized rather than the resting state?

To determine if the cross-linker, which contains a positively charged nitrogen atom, has some noncovalent effect on channel activity, a brief pulse of DMS was applied to nAChR-expressing oocytes. DMS in millimolar concentrations evokes an inward current, showing that DMS itself is a cholinergic agonist, although a very weak one (Fig. 4 *A* and *B*). Accordingly, DMS is able to compete with [^{3}H]ACh binding at nAChR-rich membranes from *Torpedo californica* ($IC_{50} \approx 20 \pm 5$ mM; Fig. 4*C*) when a very

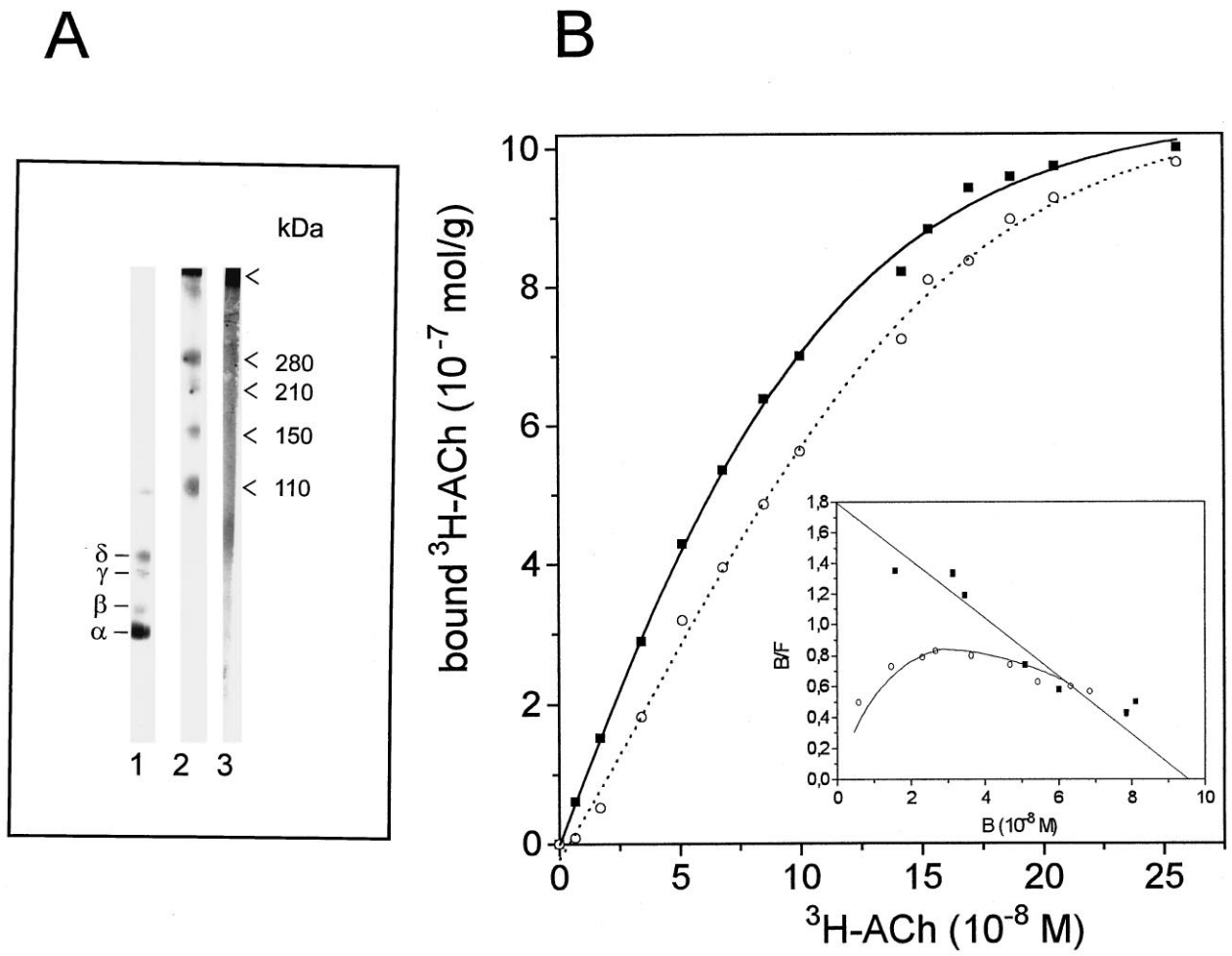

FIG. 2. Properties of the nAChR from *Torpedo californica* after cross-linking with DMS. (*A*) Gel electrophoresis (5% acrylamide) of native nAChR (lane 1), Triton X-100-solubilized nAChR cross-linked with 0.5 mg/ml DMS for 1 h (lane 2), and nAChR-rich membranes cross-linked with 2 mg/ml for 2 h (lane 3). Arrows indicate subunit dimers (≈110 kDa), trimers (≈150 kDa), tetramers (≈210 kDa), and pentamers (≈280 kDa) and polymerized nAChR (from bottom to top). No intermediate stages of cross-linking can be seen after cross-linking of membrane vesicles (lane 3). (*B*) [^{3}H]ACh binding of native receptor (○) and after cross-linking with DMS (■). (*Inset*) Scatchard plot (B, bound [^{3}H]ACh; F, free [^{3}H]ACh). The linear Scatchard plot after cross-linking indicates the loss of cooperativity (K_d = 50 nM; Hill coefficient n_H = 1.0).

short incubation time is used to minimize covalent cross-linking and hydrolysis of the cross-linker. This agonistic effect of DMS is unrelated to the cross-linking reaction, which does not cause a displacement of [^{3}H]ACh.

The short monofunctional homologue MAI also acts as a cholinergic agonist in almost the same concentration range as DMS (Fig. 4 *A* and *B*). This agonistic effect is therefore due to the positively charged imidate group and is not dependent on the length of the compound. MAI is also able to compete with [^{3}H]ACh binding ($IC_{50} \approx 50 \pm 5$ mM; Fig. 4*C*).

The products of hydrolysis of DMS and MAI, suberic acid dimethyl ester and methyl acetate, were not effective as cholinergic agonists, nor were DMS and MAI able to evoke any current in uninjected oocytes.

Lysine Modification and Intermolecular Cross-Linking Do Not Affect Allosteric Properties of the nAChR. Covalent modification of the nAChR with MAI has no effect on the shape of the [^{3}H]ACh binding curve (Fig. 5*B*). The binding curve is indistinguishable from that of native receptor. In electrophysiological experiments, modification of the oocytes with MAI reduced the ACh-evoked current by about 50%, possibly due to a reaction with lysine residues located near the cation channel, but in contrast to DMS, MAI was unable to abolish the ACh-evoked current (data not shown).

To distinguish between effects of intermolecular cross-linking (between subunits of neighboring receptors) and intersubunit cross-linking (between neighboring subunits within one receptor molecule), we measured [^{3}H]ACh binding after cross-linking with the nonagonistic BS^3. Although the spacer length of BS^3 is nearly the same as for DMS (11.4 versus 11.0 Å), this cross-linking reagent was unable to introduce intersubunit cross-links, as no intermediate stages of cross-linking were found when Triton-solubilized nAChR was used (Fig. 5*A*, lane 2). When nAChR-rich membranes of high receptor density are used, low BS^3 concentrations result in formation of subunit dimers and polymers only (no trimers, etc.; Fig. 5*A*, lane 3), whereas high BS^3 concentrations result in total receptor polymerization, due to intermolecular cross-linking (Fig. 5*A*, lane 4). The difference in cross-linking with DMS and BS^3 might be explained by the size of the *N*-hydroxysuccinimide group of BS^3, which may make cross-linking of less exposed lysine residues impossible.

No effect on [^{3}H]ACh binding was detected after BS^3 cross-linking (Fig. 5*B*). The binding curve is the same as with native receptor. Thus, cross-linking between neighboring receptor molecules (intermolecular cross-linking) is not responsible for the fixing of receptor states.

Glutardialdehyde Cross-Linking Fixes the nAChR in a State of Low [^{3}H]ACh Affinity in the Absence of Carbamoylcholine but in a State of High [^{3}H]ACh Affinity in Its Presence. According to the results with DMS, bifunctional cross-linkers that are not receptor agonists should arrest the receptor in a low-affinity state. Glutardialdehyde (another

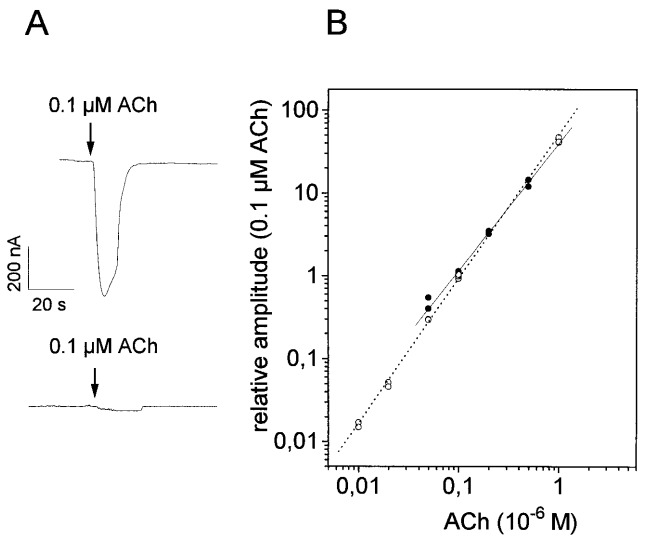

FIG. 3. Effect of DMS cross-linking on ACh-induced current at nAChRs from rat muscle expressed in *Xenopus* oocytes. (*A*) Whole-cell current induced by ACh application before (upper trace) and after (lower trace) 30 min of DMS cross-linking (<10% remaining current). The oocytes were washed for at least 10 min after DMS cross-linking, to remove excess of free cross-linker. (*B*) Dose–response curve before (○) and of the residual current after (●) 30 min of DMS cross-linking (no detectable current after 1 h). The relative amplitude is plotted against the concentration of ACh in a double-logarithmic plot. The amplitudes are normalized to the current induced by 0.1 μM ACh. (After DMS cross-linking it was not possible to record the current below 0.06 μM ACh, because the total current amplitude was below ≈8 nA).

lysine-specific cross-linking reagent) produces a cross-linking pattern similar to that of DMS (Fig. 6*A*). But, since it is not a cholinergic agonist (data not shown), it should not induce desensitization of the nAChR.

After glutardialdehyde cross-linking [^{3}H]ACh binding is biphasic (Fig. 6 *B* and *C*), with one binding site having a K_d characteristic for the receptor's high-affinity desensitized state ($K_d \approx 35 \pm 5$ nM, 12% of the total number of binding sites) and another with a low affinity for [^{3}H]ACh ($K_d \approx 2$–5 μM; see also Table 1), representing 88% of the total binding sites. Both the low- and the high-affinity receptor populations show a Hill coefficient of $n_H = 1.0 \pm 0.2$ and are therefore no longer able to make any transitions to other states (Fig. 6*C* and *Inset*). Thus, most of the receptor actually becomes fixed in the resting state, a state of low agonist affinity. The K_d is in the micromolar range and the cross-linked receptor obviously is no longer able to desensitize.

To decide if the existence of the high-affinity population is due to incomplete cross-linking, the incubation time with glutardialdehyde was increased to 36 h and fresh reagent was added after 20 h. Nevertheless the amount of the high-affinity population was not significantly reduced after this procedure (Fig. 6*C*, *Inset*).

Our experiments so far demonstrate that an agonistic (DMS) and a nonagonistic cross-linker (glutardialdehyde) fix the nAChR in a high-affinity (desensitized) and a low-affinity (resting) state, respectively. This suggests that "freezing" of the receptor's desensitized state should not be limited to the use of DMS as a cross-linking reagent, but should, in principle, also be achieved by cross-linking with (nondesensitizing) glutardialdehyde in the presence of desensitizing amounts of an agonist—e.g., carbamoylcholine.

Indeed, by cross-linking with glutardialdehyde in the presence of 0.1 mM carbamoylcholine, we achieved the same result as with DMS: [^{3}H]ACh binding affinity is enhanced after cross-linking ($K_d \approx 45 \pm 5$ nM), cooperativity is lost and maximal binding is, as expected, unaltered (Fig. 6 *B* and *D*; see also Table 1).

Table 1. Summary of the binding constants of the nAChR after cross-linking

nAChR	K_d, nM	Hill coefficient
Native	75	1.6
DMS-treated	50	1.0
Glutardialdehyde-treated	35 (high affinity)	1.0
	2,000–5,000 (low affinity)	1.0
Glutardialdehyde and carbamoylcholine	45	1.0

DISCUSSION

Our results demonstrate that it is possible to fix the nAChR in different allosteric states by chemical cross-linking. This will allow structural investigations of isolated nAChR states without inducing equilibrium shifts.

With the agonistic cross-linker DMS we were able to arrest the nAChR in a high-affinity (most probably a desensitized) state. After DMS treatment the nAChR could not return to the resting state, as shown by its Hill coefficient of $n_H = 1.0$. This effect cannot be due to the permanent presence of the agonist DMS (and noncovalent desensitization of the nAChR), because the cross-linker hydrolyzes very quickly and the product of hydrolysis, suberic acid dimethyl ester, is not a cholinergic agonist. Furthermore, the monofunctional homologue MAI, which is also a cholinergic agonist, does not cause a permanent desensitization of the receptor.

The lysine-specific, nonagonistic cross-linker BS^3 does not introduce cross-links between neighboring subunits, but cross-links neighboring receptor molecules in nAChR-rich vesicles of high receptor density. We assume that this is due to sterical hindrance of the sulfonated *N*-hydroxysuccinimide groups, which are much bigger than the imidate groups of DMS. This implies that the lysine residues leading to intersubunit cross-linking may be located in the extracellular "funnel" of the receptor near the ion channel. At least the cross-linked residues might be less exposed and not located at the receptor's surface.

The experiments with BS^3 and MAI demonstrate that the shift in the nAChR's binding characteristics after DMS cross-linking cannot be ascribed to lysine modification, intermolecular cross-linking, or a noncovalent effect of the agonistic cross-linker alone. The change in the receptor's ACh affinity is caused by a shift of the equilibrium to the high-affinity state. This is due to the activation and desensitization of the nAChR by noncovalent binding of DMS to the ACh binding site and subsequent fixation of this state by covalent cross-linking of lysine residues not located at the ACh binding site.

After glutardialdehyde cross-linking the [^{3}H]ACh binding curve is biphasic, with a receptor population of low affinity and a population of high agonist affinity. The existence of the first population seems to be due to an arrest of the receptor in the resting state. This allowed us to determine the K_d value of the receptor's resting state directly by equilibrium binding. This was not possible before because of the receptor's rapid desensitization upon equilibration with an agonist. Our results do not point to the existence of two highly different binding sites in the receptor's resting state, as has been postulated by others for the mouse muscle receptor (27) and for the *Torpedo* receptor (28), on the basis of electrophysiological measurements. If a second, much lower-affinity, binding site in the receptor's resting state does exist, we would indeed not have been able to detect it, but the extrapolation of the data in the Scatchard plot to B_{max} yields the same number of binding sites as the total number of binding sites of a control performed under the same conditions. This extrapolation should yield only 50% of the control's B_{max} if a second (lower-affinity) binding site, not covered by our data points, exists.

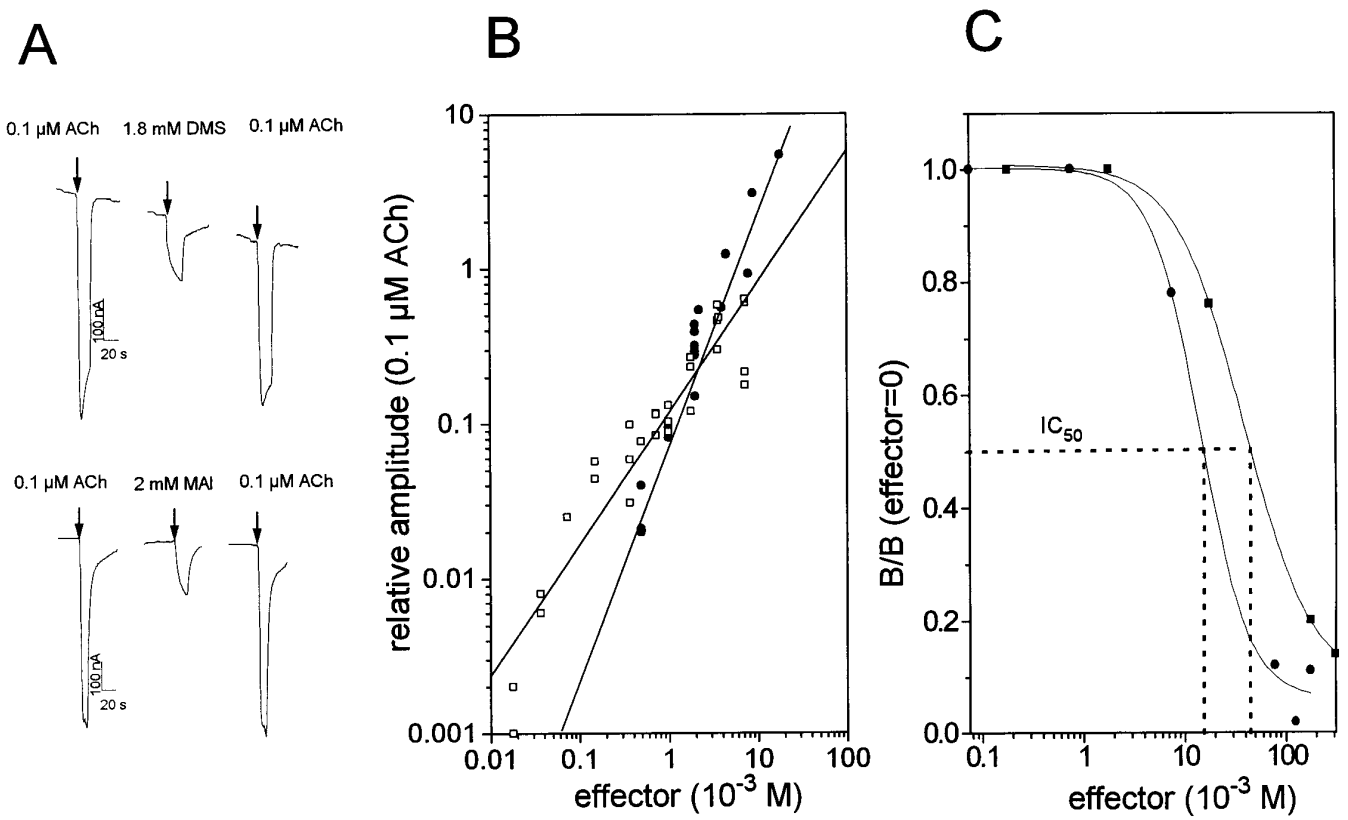

FIG. 4. Agonistic effects of the imidates DMS and MAI with rat muscle nAChR expressed in *Xenopus* oocytes. (*A*) Whole-cell current (rat muscle nAChR) induced by DMS application (upper trace) and by MAI application (lower trace). Whole-cell current induced by ACh application is shown before and after DMS/MAI application. (*B*) Dose–response curves of DMS (□) and MAI (●). The relative amplitudes are plotted against the DMS/MAI concentration in a double-logarithmic plot. The amplitudes are normalized to the response induced by 0.1 μM ACh. (*C*) Competition of DMS (●) and MAI (■) with [^{3}H]ACh at nAChR-rich membranes from *Torpedo californica*. Bound [^{3}H]ACh in the presence of DMS/MAI is normalized to bound [^{3}H]ACh in the absence of DMS/MAI and plotted against the concentration of effector (DMS/MAI).

The existence of an nAChR population of high-affinity binding after glutardialdehyde cross-linking can be explained in two different ways. Either this population represents incompletely cross-linked receptors, or some desensitized receptors exist even in the absence of agonist (29). This "predesensitized" receptor would therefore become fixed in the high-affinity state by cross-linking with glutardialdehyde. The latter explanation is supported by the finding that this population can be diminished neither by adding fresh cross-linker after several hours nor by increasing the incubation time.

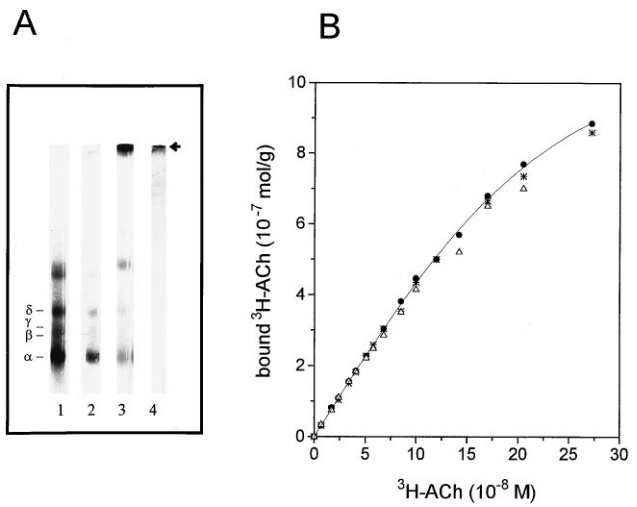

FIG. 5. Properties of the nAChR from *Torpedo californica* after cross-linking with bis(sulfosuccinimidyl) suberate (BS^3) and modification with MAI. (*A*) Gel electrophoresis of native nAChR (lane 1), Triton X-100-solubilized nAChR cross-linked with 0.5 mg/ml BS^3 for 1 h (lane 2), nAChR-rich membranes cross-linked with 1.0 mg/ml BS^3 for 1 h (lane 3), and nAChR-rich membranes cross-linked with 1 mg/ml BS^3 for 2 h. The arrow indicates polymerized nAChR, which does not enter the gel. No intermediate stages of cross-linking can be seen with Triton-solubilized nAChR, indicating the lack of intersubunit cross-links. (*B*) [^{3}H]ACh binding after modification with MAI (△) and after cross-linking with BS^3 (✳) as compared with native receptor (●).

We propose that the fixation of the nAChR in distinct allosteric states is due to restricted structural flexibility, especially at the subunit interfaces. This is in good agreement with the results by Unwin *et al.* (19), who observed changes of the quaternary structure upon desensitization. Indeed, we cannot exclude some effect of intramolecular cross-links (cross-links within one subunit) which may also occur, but it seems very unlikely that intrachain cross-links should be responsible for fixating the whole receptor molecule in a certain conformational state. Using radioactively labeled DMS, we have estimated the total number of cross-links per receptor to be about 15, with only some of them reacting with both functional groups (data not shown).

The sites of the cross-links that block allosteric transitions have not yet been localized. Since BS^3 (with its bulkier reactive group) is unable to introduce intersubunit cross-links, we do not believe that highly exposed lysine residues of, e.g., the cytoplasmic loop are involved. Moreover, the observation that similar cross-links are introduced in nAChRs expressed in *Xenopus* oocytes and in nAChR-rich membranes indicates that the most important cross-links are located in the extracellular part of the receptor, because the positively charged cross-linker DMS should be unable to pass the oocyte membrane. One possible site of the cross-linking reaction may be near the

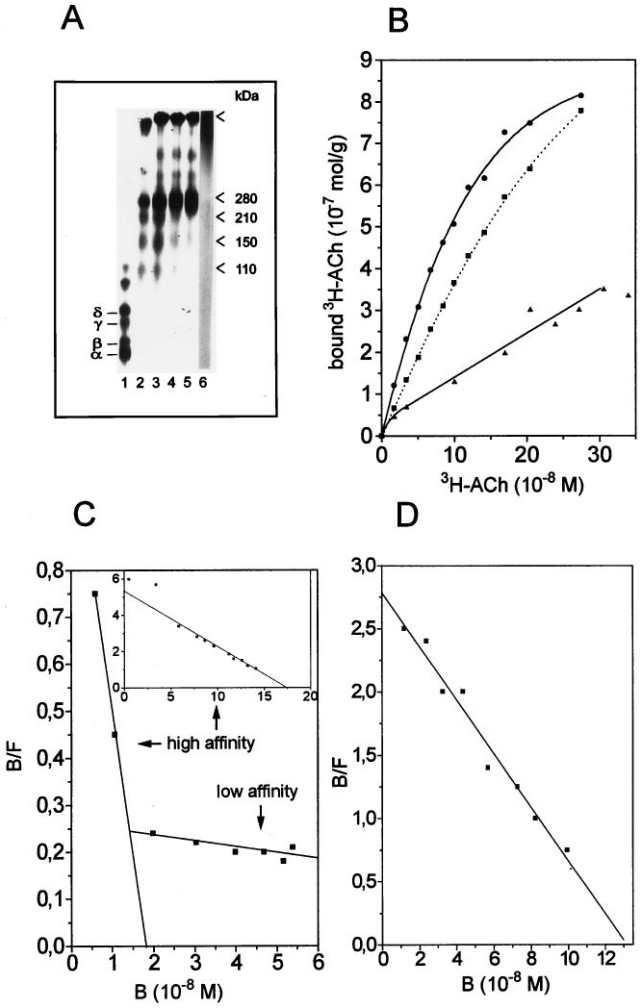

FIG. 6. Properties of the nAChR from *Torpedo californica* after cross-linking with the nonagonistic cross-linker glutardialdehyde. (*A*) Gel electrophoresis (4% acrylamide) of native nAChR (lane 1), Triton X-100-solubilized nAChR cross-linked with 0.04% glutardialdehyde for 1.5 h (lane 2), 3 h (lane 3), 8 h (lane 4), or 20 h (lane 5) and nAChR-rich membranes cross-linked with 0.04% glutardialdehyde for 20 h (lane 6). Arrows indicate subunit dimers (≈110 kDa), trimers (≈150 kDa), tetramers (≈210 kDa), and pentamers (≈280 kDa) and polymerized nAChR (from bottom to top). No intermediate stages of cross-linking can be seen after receptor cross-linking in membrane vesicles. (*B*) [^{3}H]ACh binding after cross-linking with glutardialdehyde, in the absence (▲) and in the presence (●) of carbamoylcholine as compared with native nAChR (■). Carbamoylcholine was removed before the [^{3}H]ACh binding assay and the control experiment (native receptor plus carbamoylcholine) was performed under the same conditions. (*C*) Scatchard plot after cross-linking with glutardialdehyde in the absence of carbamoylcholine. A low-affinity binding site ($K_d = -1/m$ = 2–5 μM; n_H = 1.0) and a high-affinity binding site can be seen. (*Inset*) Scatchard plot of the high-affinity binding site alone after glutardialdehyde cross-linking, as measured by using a much higher amount of protein to achieve higher concentrations of bound [^{3}H]ACh (K_d = 35 nM; n_H = 1.0). The low-affinity binding site cannot be seen under these conditions. (*D*) Scatchard plot after cross-linking with glutardialdehyde in the presence of carbamoylcholine (K_d = 45 nM; n_H = 1.0).

ion channel, where binding sites for the noncompetitive blockers [^{3}H]triphenylmethylphosphonium$^+$ [δ-subunit Ser-262 (30, 31)] and [^{3}H]chlorpromazine [β-subunit Leu-257, δ-subunit Ser-262 (32, 33)] have been identified. A lot of conserved lysine residues are indeed located in the receptor's extracellular part—in the region between the agonist-binding site and the ion channel. This region (especially the M2-M3 loop) has been shown to be rather important for the coupling between ligand binding and channel gating for different members of the family of ligand-gated ion channels (34–36).

One possible effect of interface cross-linking might concern the agonist-binding site directly, because the ACh-binding site is located at the subunit interfaces. It has been stated that the distance between the binding site disulfide on the α-subunit and the negative binding subsite on the neighboring γ- and δ-subunit might change upon receptor activation (18, 37). Thus, cross-linking at the subunit interfaces might prevent such conformational changes. If such a distance change is essential for the coupling of agonist binding and channel gating, this would explain the fixing of different receptor states by covalent interface cross-linking.

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