Nonequivalence of α-Bungarotoxin Binding Sites in the Native Nicotinic Receptor Molecule†

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ABSTRACT: In the native, membrane-bound form of the nicotinic acetylcholine receptor (M-AcChR) the two sites for the cholinergic antagonist α-bungarotoxin (α-BGT) have different binding properties. One site has high affinity, and the M-AcChR/α-BGT complexes thus formed dissociate very slowly, similar to the complexes formed with detergent-solubilized AcChR (S-AcChR). The second site has much lower affinity ($K_D \approx 59 \pm 35$ nM) and forms quickly reversible complexes. The nondenaturing detergent Triton X-100 is known to solubilize the AcChR in a form unable, upon binding of cholinergic ligands, to open the ion channel and to become desensitized. Solubilization of the AcChR in Triton X-100 affects the binding properties of this second site and converts it to a high-affinity, slowly reversible site. Prolonged incubation of M-AcChR at 4 °C converts the low-affinity site to a high-affinity site similar to those observed in the presence of Triton X-100. Although the two sites have similar properties when the AcChR is solubilized in Triton X-100, their nonequivalence can be demonstrated by the effect on α-BGT binding of concanavalin A, which strongly reduces the association rate of one site only. The B_{max} of α-BGT to either Triton-solubilized AcChR or M-AcChR is not affected by the presence of concanavalin A. Occupancy of the high-affinity, slowly reversible site in M-AcChR inhibits the Triton X-100 induced conversion to irreversibility of the second site. At difference with α-BGT, the long α-neurotoxin from *Naja naja siamensis* venom (α-NTX) binds with high affinity and in a very slowly reversible fashion to two sites in the M-AcChR (Conti-Tronconi & Raftery, 1986). We confirm here that Triton-solubilized AcChR or M-AcChR binds in a very slowly reversible fashion the same amount of α-NTX. In support of the contention that α-BGT binds "irreversibly" to one site only in the native M-AcChR, we found that when M-AcChR is saturated with radiolabeled α-BGT, addition of α-NTX markedly accelerates the dissociation of the bound α-BTX, presumably because occupancy of the second site by tightly bound α-NTX influences and decreases the affinity for α-BGT of the other site. The different properties of the two α-BGT binding sites in the native AcChR molecule support the possibility that these sites have different structural properties and that a sugar moiety is in close proximity to at least one such site.

The nicotinic acetylcholine receptor (AcChR) of peripheral tissues such as *Torpedo* electroplax and mammalian muscle is a transmembrane protein formed by homologous subunits in a stoichiometry $\alpha_2\beta\gamma\delta$ [reviewed in Conti-Tronconi and Raftery (1982), McCarthy et al. (1986), and Maelicke (1987)]. Binding of cholinergic agonists to two low-affinity sites, which operate in a somehow cooperative fashion, results in a fast conformational change and in activation of a cation channel contained in the AcChR molecule (Conti-Tronconi & Raferty, 1982; McCarthy et al., 1986). Ligand binding to two high-affinity sites causes a slow conformational change that results in a desensitized state, characterized by inability of the channel to open (Katz & Thesleff, 1957; Rang & Ritter, 1970a,b). A major goal in the elucidation of AcChR function has been to define the ligand binding events leading to activation and inactivation of the cation channel. Two models for ligand binding mechanisms have been proposed.

In linear models [reviewed in Sine and Taylor (1980), Conti-Tronconi and Raftery (1982), and Maelicke (1987)] activation and desensitization occur upon binding to the same two sites which undergo multiple changes in affinity as a consequence of ligand binding. Strong support of this model is the demonstration that each of the two α-subunits present in the AcChR molecule contains a high-affinity binding site for the affinity label bromoacetylcholine (Wolosin et al., 1980) and that the peptide cholinergic antagonist α-bungarotoxin (α-BGT),[1] which also binds to two sites [reviewed in Conti-Tronconi and Raftery (1982)], recognizes the denatured α-subunit (Haggerty & Froehner, 1981; Gershoni et al., 1983; Wilson et al., 1984). It has been therefore concluded that also the two sites for α-BGT are on the two α-subunits, and because α-BGT irreversibly inhibits AcChR activation, this has been construed as proof that activation and desensitization are controlled by the same two sites on the α-subunits.

A second model, a multiple-site model, argues that separate low- and high-affinity sites regulate channel activation and receptor desensitization (Dunn et al., 1983; Dunn & Raftery, 1982a,b; Raftery et al., 1983). Binding of acetylcholine and carbamoylcholine causes a conformational change of the AcChR molecule which results in enhancement of the fluorescence of the fluorescent probe 4-[[*N*-[(iodoacetoxy)-ethyl]-*N*-methyl]amino]-7-nitrobenz-2-oxa-1,3-diazole (IANBD) covalently attached to the AcChR molecule itself. This process occurs on a time scale compatible with the fast process leading to channel opening (Dunn & Raftery, 1982a,b; Dunn et al. 1983), and the apparent dissociation constants for

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[1] Abbreviations: M-AcChR, membrane-bound acetylcholine receptor; S-AcChR, detergent-solubilized acetylcholine receptor; α-BGT, α-bungarotoxin; ^{125}I-α-BGT, α-bungarotoxin radiolabeled with ^{125}I; α-NTX, α-najatoxin; Con A, concanavalin A; SDS, sodium dodecyl sulfate; rpm, revolution per minute.

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the cholinergic binding for different cholinergic agonists of the site monitored by the changes in IANBD fluorescence are identical with the apparent equilibrium constants for activation of monovalent cation flux (Dunn & Raftery, 1982a,b). It was therefore concluded that the enhancement in IANBD fluorescence monitored binding to cholinergic site(s) involved with activation of the ion channel, because it had the affinity for cholinergic ligands characteristic of activation of the resting state of the AcChR, and triggered a conformational change fast enough to be within the chain of events ultimately leading to channel opening (Dunn & Raftery, 1982a,b). The agonist-induced enhancement of IANBD fluorescence was seen under equilibrium conditions when, according to the linear model, the receptor should be densitized. These observations suggest that low-affinity binding sites exist that are separate from the high-affinity site that regulates receptor desensitization. Further evidence to support this possibility comes from the observation that enhancement of IANBD fluorescence induced by cholinergic agonists is also observed when both binding sites on the α-subunits are covalently modified by bromoacetylcholine (Conti-Tronconi et al., 1982; Dunn et al., 1983).

α-BGT interaction with the AcChR has been crucial for constructing models of its function. The long α-neurotoxins from the venom of Elapid snakes, like α-BGT from *Bungarus multicinctus* venom and α-cobratoxin (α-NTX) from the venom of different *Naja* species, form a family of peptide cholinergic antagonists that have been very useful for isolation and characterization of AcChRs from different peripheral tissues, like mammalian muscle and fish electric organ [reviewed in Klett et al. (1973), Blanchard et al (1979), Sine and Taylor (1980), Wang and Schmidt (1980), Kang and Maelicke (1980), and Chang et al. (1984)]. These toxins binding specifically and with high affinity to the AcChR. They form complexes that dissociate very slowly (and cause an irreversible block of the AcChR in functional assays). For these reasons, although their binding to the AcChR is actually not irreversible or covalent, the binding of α-BGT and α-NTX to AcChR is sometimes referred to as irreversible. The kinetic studies focused on α-BGT binding to membrane-bound AcChR yielded conflicting results, and the association kinetics of this toxin to M-AcChR have been reported to be monophasic (Blanchard et al., 1979; Lukas et al., 1981) or multiphasic (Leprince et al., 1981); its dissociation has been shown to occur over several hours (Lukas et al., 1981) or days (Blanchard et al., 1979). Most studies on the properties of α-BGT binding to *Torpedo* AcChR have assumed the linear model, and they have been carried out with detergent-solubilized AcChR [reviewed in Conti-Tronconi and Raftery (1982) and Chang et al. (1984)] or with AcChR-rich membranes isolated by using protocols in which proteases were not inhibited (Lukas et al., 1981; Leprince et al., 1981). Under these circumstances, the two α-BGT sites have very similar binding properties, and this has lent further credence to the notion of two identical binding sites on identical domains. However, interaction with nondenaturing detergents drastically affects the binding properties of AcChR for cholinergic agonists [reviewed in Conti-Tronconi and Raftery (1982)], and (at least in mammalian brain) proteolysis affects the affinity of the AcChR for nicotine (Lippiello & Fernandes, 1986). Because most studies on the interaction between α-BGT and *Torpedo* AcChR have been carried out under circumstances when artifactual changes in the binding properties may have been present because of the effects of detergents and proteases, we have investigated some binding properties of the two α-BGT binding sites in the nonproteolized, membrane-bound *Torpedo* AcChR (M-AcChR) and compared them with those of AcChR solubilized in the nondenaturing detergent Triton X-100 (S-AcChR).

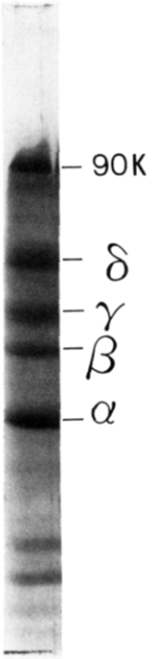

FIGURE 1: SDS gel electrophoresis pattern of a typical *Torpedo* membrane preparation enriched in AcChR, after treatment at pH 11. The four AcChR subunits (α, β, γ, δ) are the main protein constituent. A contaminant protein of approximate M_r 90 000 is also present. The faint protein bands of low M_r are degradation products of the AcChR subunits.

Materials and Methods

Preparation of AcChR-Rich Membrane Fragments. AcChR-rich membrane fragments were prepared from *Torpedo californica* electric organs (Elliot et al., 1980) and extracted at pH 11 to remove nonreceptor proteins (Neubig et al., 1979; Elliot et al., 1979). In this method Ca^{2+}-dependent and thiol-dependent proteases are fully inhibited, and the AcChR present in the membrane fragments thus obtained is intact. Upon SDS gel electrophoresis (Laemmli, 1970) these preparations (M-AcChR) showed the four AcChR subunits as the major protein components (Figure 1). The specific activity of these preparations (expressed as nanomoles of α-BGT binding sites/milligram of protein), measured in the presence of Triton X-100, was 4–7 nmol/mg of protein, which is close to the maximum theoretical activity of pure AcChR (7.2 nmol/mg). The AcChR-rich membrane fragments isolated from *Torpedo* electric organ can spontaneously reseal to form microsacs. When this occurs, more than 95% of the closed vesicles thus formed are right-side-out [see below and Hartig and Raftery (1979); Strader & Raftery, 1980; Froener, 1980; St. John et al., 1982; Conti-Tronconi et al., 1982]. The M-AcChR preparations were stored at 4 °C and used within 1 week. For some experiments, M-AcChR was left at 4 °C for up to 3 weeks.

Purification and Radiolabeling of Snake Toxins and Calibration of ^{125}I-α-BGT. α-BGT was purified from *B. multicinctus* venom (Biotoxins Inc.), and α-cobratoxin (α-NTX) was purified from *Naja naja siamensis* venom (Biotoxins Inc.) following the procedure of Ong and Brady (1974). Toxin purity was assessed by SDS gel electrophoresis (Laemmli, 1970) using an exponential gradient of polyacrylamide (8–20%) and by amino-terminal amino acid se-

quencing using a gas-phase sequenator (Applied Biosystems). Only the known sequences of α-BGT or of α-NTX were found. The purified toxins were radiolabeled with ^{125}I by using chloramine T according to the procedure of Lindstrom et al. (1981), which yields monoiodinated toxins (Lindstrom et al., 1981), with the following modification: after completion of the iodination reaction, any remaining chloramine T was reduced by adding an excess of sodium metabisulfite, and 25 μL of a saturated solution of potassium iodide was added. The iodinated protein was separated from the unreacted iodine and the other low molecular weight components by use of a desalting column (Pierce, 5 mL of settled bed). The specific activity of ^{125}I-α-BGT was calibrated as described in Blanchard et al. (1979).

Assay of ^{125}I-α-BGT and ^{125}I-α-NTX Binding: DEAE Disk Assay. The concentration of irreversible ^{125}I-α-BGT and ^{125}I-α-NTX binding sites was measured by the DEAE disk assay of Schmidt and Raftery (1973). The disk assay was carried out either in the presence of 0.1% Triton X-100 during both the incubation of AcChR with the radiolabeled toxin and the washings or without any detergent. Previous experiments (Conti-Tronconi & Raftery, 1986) showed that M-AcChR binds quantitatively to DEAE disks. In some experiments, after the incubation was carried out without detergent, the last washings were done with buffer containing 0.1% Triton X-100.

Assay of ^{125}I-α-BGT Binding: Centrifugation Assay. The total irreversible plus reversible binding site concentration was measured by a centrifugation assay. A constant amount of M-AcChR (32–50 nM) was incubated with increasing concentrations of ^{125}I-α-BGT (up to 300 nM) at room temperature for 60 min in 10 mM phosphate buffer and 50 nM NaCl, pH 7.2, in a total volume of 250 μL. Parallel control aliquots for nonspecific ^{125}I-α-BGT binding were prepared, preincubated with a 10-fold excess of unlabeled α-BGT, which was left in the mixture throughout the whole experiment. After incubation, 10-μL aliquots from each tube were counted for radioactivity, and the remainder was centrifuged for at least 30 min at 8000 rpm in a Sorvall SHMT rotor. Three 10-μL aliquots of supernatant were counted for radioactivity. The amount of bound toxin was calculated from the difference between the control and the sample aliquots after centrifugation.

In another set of experiments, a centrifugation assay was carried out by using the same conditions used for the disk assays, which were also carried out with or without Triton X-100 on the same membrane preparation. Increasing volumes of M-AcChR suspension (50–70 nM) were incubated for 60 min at room temperature with a constant concentration of ^{125}I-α-BGT (500 nM in a final volume of 250 μL). Parallel control aliquots for nonspecific ^{125}I-α-BGT were preincubated with a 10-fold excess of unlabeled α-BGT, which was left in the mixture throughout the whole experiment. both the sample and the control aliquots were processed as described above, and the α-BGT binding site concentration was obtained from the difference in the radioactivity remaining in the supernatant between the control and the sample.

Assessment of the Effect of Irreversibly Bound α-BGT on the Further Binding to the Reversible Site. Aliquots of M-AcChR (approximately 100 μL of a 5 μM suspension) were preincubated with a 10-fold excess of unlabeled α-BGT for 60 min at room temperature. The membrane fragments were washed free of unbound α-BGT by dilution to 30 mL and centrifugation in a Sorvall SA 600 rotor at maximum speed for 45 min at 6 °C. The pelleted membranes were resuspended, and the wash was repeated a second time. A control aliquot of M-AcChR was incubated without α-BGT and then diluted and centrifuged as described above. The M-AcChR pellets were resuspended in the centrifugation assay buffer, diluted to the necessary concentrations in the appropriate buffer (see above), and used either for DEAE disk assays, carried out with and without Triton X-100, or for a centrifugation assay. In the centrifugation assay, 500 μL of a suspension of 50 nM M-AcChR was incubated with increasing amounts of ^{125}I-α-BGT for 1 h at room temerature. The membranes containing the AcChR were pelleted by centrifugation for 45 min at 8000 rpm in a Sorvall SHMT rotor and counted for bound radioactivity. Control aliquots for nonspecific ^{125}I-α-BGT binding were preincubated with a 100-fold excess of unlabeled α-BGT, which was left in the mixture throughout the experiment, and processed as described above.

Assessment of the Effect of Bound Concanavalin A on ^{125}I-α-BGT Binding. The influence of bound concanavalin A (Con A) on ^{125}I-α-BGT binding was assessed as follows. The effect on total ^{125}I-α-BGT binding to either S-AcChR or M-AcChR was tested by incubating for 1 h at room temperature aliquots of M-AcChR (approximately 0.1 μM, measured as α-BGT binding sites) in 10 mM sodium phosphate buffer, pH 7.0, with 2-fold and 10-fold molar excesses of Con A over the AcChR subunits (Con A concentrations of 0.5 and 2.5 μM, respectively) or without Con A. The α-BGT binding sites present in the Con A treated sample and on the untreated control sample were measured by DEAE disk assay, in the presence or absence of Triton X-100. In the case of the Con A treated samples the buffers used in the disk assays contained the same Con A concentration as during the preincubation. The final concentrations in the assay were 20–80 nM AcChR, 150 nM ^{125}I-α-BGT, and 0.5, 2.5, or 0 μM Con A.

To test the effect of bound Con A on the time course of ^{125}I-α-BGT binding to S-AcChR, 20-μL aliquots of 3 μM M-AcChR (measured as α-BGT binding sites) in 10 mM sodium phosphate buffer, pH 7.0, were incubated for 30 min at room temperature with a 10-fold molar excess of Con A or without any Con A. The M-AcChR was solubilized and diluted to 1.25 nM by using 10 mM sodium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 (wash buffer) and 12.5 μM Con A. A proper volume of a solution of ^{125}I-α-BGT in the same buffer was added. The final concentrations were 1 nM S-AcChR, 2 nM ^{125}I-α-BGT, and 10 or 0 μM Con A. The mixture was incubated at room temperature for 2 h. In some experiments the incubation was prolonged up to 21 h. At different time intervals during the incubation 100-μL aliquots were pipetted in triplicate onto DEAE disks, and the disks were washed three times in a large volume of wash buffer. In some experiments the wash buffer used for the washings contained 10 μM Con A. The amount of bound ^{125}I-α-BGT was measured in a Beckman γ counter. The data obtained in the absence of Con A were fitted to a simple rate equation

$$\text{fmol bound} = \text{Amp}(1 - e^{-k1_{appt}}) \quad (1)$$

where Amp is the maximum bound α-BGT. The data obtained in the presence of Con A were fitted to the double-exponential equation

$$\text{fmol bound} = \tfrac{1}{2}\text{Amp}[(1 - e^{-k1_{appt}}) + (1 - e^{-k2_{appt}})] \quad (2)$$

Effect of α-NTX on the Dissociation Rate of α-BGT/AcChR Complexes. Complexes between ^{125}I-α-BGT and M-AcChR were formed by incubating M-AcChR (1–14 μM, measured as α-BGT binding sites, in 10 mM phosphate buffer, pH 7.0) for 1 h at 6 °C with a 2-fold excess of radiolabeled ^{125}I-α-BGT. The unreacted toxin was washed away by diluting

Table I: Irreversible Binding of ^{125}I-α-BGT to AcChR-Rich *Torpedo* Membrane Fragments before and after Solubilization with Triton X-100[a]

preparation	before Triton X-100 (nmol/mL)	after Triton X-100 (nmol/mL)
1	7.90	15.71
2	5.40	9.85
3	4.60	9.10
4	4.20	9.30
5	9.5	19.10
6	5.6	11.30
7	8.05	16.15

[a] The binding was measured by DEAE disk assay as described under Materials and Methods.

the reaction mixture with 30 mL of the same buffer and pelleting the membranes at 19000 rpm for 20 min in a SS-34 Sorvall rotor. The washed complexes were resuspended in approximately 300 μL of the same buffer, and aliquots of 0.1–0.8 nmol of α-BGT/M-AcChR complexes (measured as α-BGT binding sites) were incubated at room temperature with an excess of either unlabeled α-BGT (8-fold excess in two experiments, 7-fold excess in one experiment) or α-NTX (8-fold excess in two experiments, 10-fold excess in one experiment). The final M-AcChR concentration (measured as α-BGT binding sites) was 1 μM. Aliquots of the mixtures were taken at time intervals and pipetted onto DEAE disks. The disks were washed as described by Schmidt and Raftery (1973), and the ^{125}I-α-BGT still bound was measured in a Beckman γ counter. Because the dissociation rate of α-BGT/AcChR is very slow [reviewed in Conti-Tronconi and Raftery (1982)], in preliminary experiments the half-life of complexes between M-AcChR or S-AcChR and ^{125}I-α-BGT was determined by measuring the remaining ^{125}I-α-BGT/AcChR complexes at several time intervals up to 40 h. For the experiments aimed at determining the half-life of the complexes in the presence of α-NTX, which, as will be described below, greatly accelerates the dissociation of the complexes, data points were collected from time 0 up to 5 h. Also in these experiments parallel curves of the dissociation of the complexes in the presence of an excess of unlabeled α-BGT were determined. The dissociation rate of the ^{125}I-α-BGT/AcChR complexes was calculated from the amount of ^{125}I-α-BGT still bound to the AcChR at the various time intervals by using an exponential fitting program.

Results

Measurements of Irreversible ^{125}I-α-BGT Binding Sites by DEAE Disk Assay. In the disk assay of Schmidt and Raftery (1973), as well as in all the other assays that involve slow washing, gradient centrifugation, or chromatographic separation of the bound from the free toxin, only irreversible or slowly reversible complexes of α-BGT with AcChR are measured. Table I reports the results obtained with seven typical preparations in the presence and absence of Triton X-100. Consistently, M-AcChR irreversibly bound approximately 50% of the toxin bound by S-AcChR.

Occasionally (2 of 13 experiments), similar numbers of sites were present in the presence or absence of Triton. SDS–PAGE analysis of the membranes used for these experiments revealed a peptide pattern consistent with subunit proteolysis. Therefore, we tested the effects of endogenous proteolysis. M-AcChR, which, when freshly prepared, had half as many irreversible binding sites as in the presence of Triton X-100, was left at 6 °C for 18 days and tested again. The number of irreversible ^{125}I-α-BGT binding sites of M-AcChR increased to 70% of those found in the presence of Triton X-100.

Table II: Irreversible Binding of ^{125}I-α-NTX to AcChR-Rich *Torpedo* Membrane Fragments before and after Solubilization with Triton X-100[a]

preparation	before Triton X-100 (cpm × 10^{-3}/mL)	after Triton X-100 (cpm × 10^{-3}/mL)
1	16550	17500
2	21000	20900
3	21210	14490
4	17475	16225
4	15050	16750
5	13900	13700

[a] The binding was measured by DEAE disk assay as described under Materials and Methods.

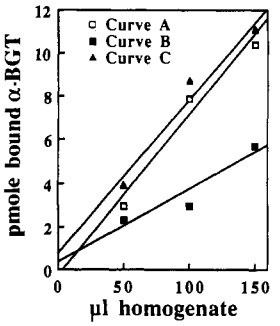

FIGURE 2: Increasing amounts of AcChR-rich membrane fragments dissolved in Triton X-100 (S-AcChR, open squares, curve A) or not [M-AcChR, triangles (curve C) and black squares (curve B)] were incubated with excess (approximately 500 nM) ^{125}I-α-BGT. For the samples indicated by open and black squares the amount of bound ^{125}I-α-BGT was measured by DEAE disks, for samples indicated by triangles by centrifugation assay. The values obtained for S-AcChR by disk assay and for M-AcChR by centrifugation assay are identical, while only half as many α-BGT binding sites were revealed for M-AcChR when the disk assay was used.

Measurement of Irreversible ^{125}I-α-NTX Binding Sites by DEAE Disk Assay. The amount of ^{125}I-α-NTX bound by M-AcChR and S-AcChR, measured by DEAE disk assay, was the same, irrespective of the presence of Triton X-100 during the incubation and the washings. Table II reports the results obtained with five different AcChR preparations. For one preparation (no. 4) the assays were carried out in two different experiments, with identical results. The values are expressed as counts per minute per microliter of M-AcChR suspension (or S-AcChR solution) instead of micromoles per milliliter because of difficulties encountered in the calibration of radiolabeled α-NTX. The difficulties in assessing the exact specific activity of ^{125}I-α-NTX, and as a consequence in measuring the molar ratio of its binding to M-AcChR and S-AcChR, were not a problem because the stoichiometry of the very slowly reversible binding of α-NTX to M-AcChR is known from direct measurements by quantitative amino-terminal sequencing of the stoichiometry of the peptides present in the α-NTX/M-AcChR complexes after extensive washings, and it is two molecules of α-NTX for one molecule of AcChR (Conti-Tronconi & Raftery, 1986). α-NTX therefore binds to both S-AcChR and M-AcChR with the same stoichiometry, i.e., two sites on each AcChR molecule, and binding to both sites is irreversible.

Measurement of Total ^{125}I-α-BGT Binding Sites by Centrifugation Assay. Figure 2 shows the results of toxin binding assays where increasing volumes of 66 nM AcChR dissolved in Triton X-100 (S-AcChR, curve A) or not (M-AcChR, curves B and C) were incubated for 60 min at room temperature with an excess (approximately 500 nM) of ^{125}I-α-BGT. For samples A and B the amount of bound ^{125}I-α-BGT was measured by DEAE disks and for sample C, by centrifugation assay. The values obtained for S-AcChR by disk

Table III: Effect of Con A on Irreversible Binding of ^{125}I-α-BGT to Triton-Solubilized (S-AcChR) or Membrane-Bound (M-AcChR) AcChR[a]

	S-AcChR (nmol/mL)			M-AcChR (nmol/mL)		
preparation	no Con A	2× Con A	10× Con A	no Con A	2× Con A	10× Con A
1	8.17	9.06	7.63	ND	ND	ND
2	9.45	10.20	9.54	ND	ND	ND
3	9.68	8.64	8.64	ND	ND	ND
4	8.25	9.87	9.80	4.30	5.43	5.25

[a] The binding was measured by DEAE disk assay as described under Materials and Methods.

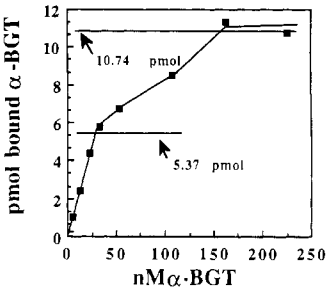

FIGURE 3: Measurement of ^{125}I-α-BGT bound to M-AcChR by centrifugation assay. Aliquots (250 μL) containing 10.74 pmol of M-AcChR, measured as α-BGT binding sites in the presence of Triton X-100, were incubated with increasing concentrations of ^{125}I-α-BGT. The number of sites of this membrane preparation measured by disk assay without Triton X-100 was exactly 50% of those measured in the presence of the detergent (inset, bars A and B). In the centrifugation assay, the amount of bound ^{125}I-α-BGT increases linearly up to the point where the ^{125}I-α-BGT concentration is approximately equivalent to half of the total concentration of binding sites, yielding a titration curve of the high-affinity sites. The slope then decreases and reaches saturation at approximately the concentration of the sites measured for S-AcChR (inset, bar C).

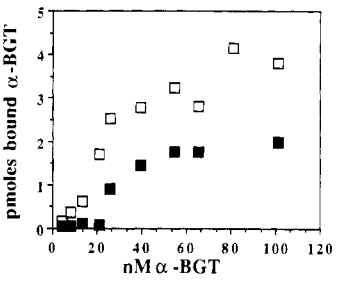

FIGURE 4: Presence of residual reversible binding sites, measured by centrifugation assay in a preparation of M-AcChR pretreated with unlabeled α-BGT and washed free of the unreacted reversibly bound toxin. The samples thus treated (black squares) still bind approximately 50% of the toxin bound by the control, untreated samples (open squares). Unspecific binding has been subtracted from both curves. See text for experimental details.

assay and for M-AcChR by centrifugation assay are identical, while only half as many α-BGT binding sites were detected for M-AcChR by the disk assay (curve B). Therefore, one of the two α-BGT sites present on M-AcChR is not irreversible and can be detected only by the centrifugation assay.

The dose dependency of α-BGT binding to this reversible site was investigated. Samples containing a constant concentration of M-AcChR (30–50 nM) were incubated with increasing concentrations of ^{125}I-α-BGT. The amount of bound ^{125}I-α-BGT was measured by centrifugation assay. The results of a typical experiment are shown in Figure 3. In this experiment 10.74 pmol of M-AcChR (measured as α-BGT binding sites in the presence of Triton X-100) in 250 μL were incubated for 60 min at room temperature with up to 225 nM α-BGT. The number of binding sites of this membrane preparation measured by disk assay without Triton X-100 was 50% of those measured in the presence of the detergent (10 and 19.3 nmol/mL, respectively). In the centrifugation assay, the amount of bound ^{125}I-α-BGT increases linearly up to the point where the ^{125}I-α-BGT concentration is approximately equivalent to half of the total concentration of binding sites (50% of 10.74 pmol in 250 μL, i.e., 21.5 nM), yielding a titration curve of the high-affinity sites. The slope then decreases, producing a plateau approximately at the expected concentration of the irreversible sites, and the binding reaches saturation at the concentration of the sites measured for S-AcChR. The biphasic behavior shown in Figure 3 was evident in most but not all experiments, and it is probably due to the different association rates of the two sites. The reversible site seems to have a slower association rate, and 1 h of incubation may not always be enough to reach equilibrium at the lower toxin concentrations.

An approximate estimate of the affinity of the reversible site was obtained by subtracting from both the total and the bound ^{125}I-α-BGT the component due to binding to the first irreversible site and calculating from the plots thus obtained the concentration of ^{125}I-α-BGT necessary to achieve half-saturation. The values obtained in six different experiments were 80, 75, 50, 18, 108, and 23 nM (average 58 ± 35 nM).

Effects of Occupancy of the High-Affinity Site on Binding to the Second Site. M-AcChR was preincubated either in the 10-fold excess of unlabeled α-BGT or without any toxin, and after removal of the unreacted toxin, disk assays were performed on both samples in the absence and presence of Triton X-100. The disk assay carried out on the untreated sample in the absence of any detergent revealed 50% of the sites measured after Triton X-100 solubilization. No ^{125}I-α-BGT bound to the unlabeled toxin treated samples, even after Triton X-100 solubilization. When the presence of residual reversible binding sites was measured by centrifugation assay, the unlabeled toxin treated samples still bound approximately 50% of the ^{125}I-labeled toxin bound by the control, untreated samples (Figure 4: open squares, control untreated samples; black squares, unlabeled toxin treated samples).

Effects of Bound Con A on α-BGT Binding. To differentiate between the two α-BGT sites on S-AcChR, we investigated the effect of bound Con A on ^{125}I-α-BGT binding. In four different experiments, preincubation with up to 2.5 μM Con A did not change the total amount of ^{125}I-α-BGT bound to S-AcChR (Table III). Bound Con A did not reduce the amount of ^{125}I-α-BGT irreversibly bound to M-AcChR (Table III). The effect on the rate of association of ^{125}I-α-BGT to S-AcChR was then investigated by using much lower concentrations of both S-AcChR and ^{125}I-α-BGT (1 and 2 nM, respectively, compared with 80 and 150 nM in the disk assay), so that the binding reaction was complete after approximately 60 min. In Figure 5 the results of a typical experiment are shown and fitted, by use of an exponential fitting program, to a single-exponential equation in the case of the binding isotherm obtained in the absence of Con A (Triangles in Figure 6) and to a double-exponential equation in the case of the binding isotherm obtained in the presence of 10 μM Con A (circles in Figure 6), as described under Materials and Methods. In the absence of Con A the calculated maximum amount of bound α-BGT was 37.5 ± 0.58 fmol, with a rate

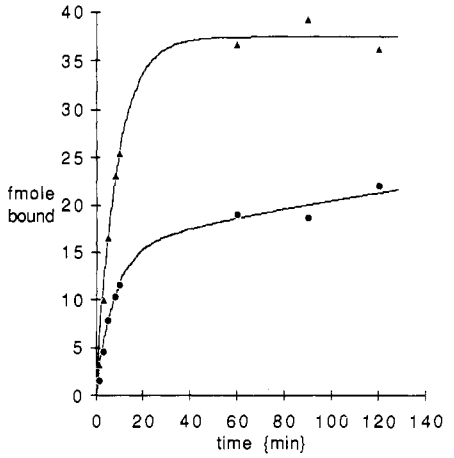

FIGURE 5: Effect of Con A on the rate of association of ^{125}I-α-BGT to S-AcChR. S-AcChR (1 nM) was incubated with 2 nM α-BGT for 2 h with 10 μM Con A (circles) or without any Con A (triangles). At different time intervals, aliquots were taken and the amount of bound ^{125}I-α-BGT measured by DEAE disk assay. The data were fitted by using an exponential fitting program to a single-exponential equation in the case of the curve obtained in the absence of Con A (triangles) and to a double-exponential equation for the data obtained in the presence of 10 μM Con A (circles), as described under Materials and Methods. In the absence of Con A the calculated maximum amount of bound α-BGT was 37.5 ± 0.58 fmol, with a rate of association of 0.114 ± 0.005 min^{-1}. In the presence of Con A the calculated maximum amount of bound α-BGT was 30.3 ± 6.69 fmol, and the association rates were 0.130 ± 0.040 min^{-1} for a fast associating site accounting for 50% of the binding and 0.004 ± 0.005 min^{-1} for a slow associating site accounting for the remaining binding.

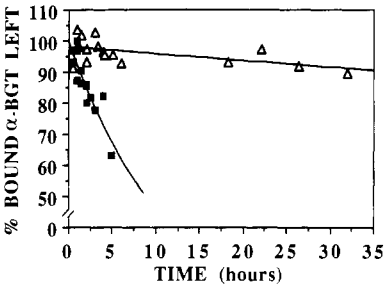

FIGURE 6: Effect of the presence of an excess of unlabeled α-NTX (black squares) or α-BGT (open triangles) on the dissociation rate of complexes formed between ^{125}I-α-BGT and M-AcChR. Each curve resports the results obtained in three independent experiments, carried out with different preparations of both M-AcChR and toxins. The concentrations used were the following: M-AcChR/^{125}I-α-BGT complexes, 1 μM; α-NTX; 8 μM in two experiments and 10 μM in one experiment; α-BGT, 8 μM in two experiments and 7 μM in one experiment. Both the consistency and the magnitude of the effect are evident. The rate of dissociation in the presence of unlabeled α-BGT is very slow ($t_{1/2}$ for this curve equals 290 h) and it is markedly accelerated by the presence of α-NTX ($t_{1/2}$ for this curve equals 8.75 h; correlation coefficient, 0.79). See text for further details.

of association of 0.114 ± 0.005 min^{-1}. In the presence of Con A the calculated maximum amount of bound α-BGT was 30.3 ± 6.69 fmol, and the association rates were 0.130 ± 0.040 min^{-1} for a fast associating site accounting for 50% of the binding and 0.004 ± 0.005 min^{-1} for a slow associating site accounting for the remaining binding. In two experiments the incubation was continued up to 21 h, and, as expected, it was found that the sample incubated with Con A had reached 97 and 105% of the values obtained in the absence of Con A, respectively.

Effect of α-NTX on the Dissociation Rate of α-BGT/AcChR Complexes. The basal dissociation rate of the irreversible complexes between M-AcChR and ^{125}I-α-BGT was measured in the presence of an excess (7-fold) of unlabeled α-BGT for incubation periods of up to 32 h. The half-life of the complexes was found to be >250 h, in good agreement with the accepted notion that this toxin forms very slowly reversible complexes (Blanchard et al., 1979). When an 8–10-fold excess of unlabeled α-NTX was present, the dissociation rate was markedly enhanced. The half-lives of the complexes obtained in three independent experiments were 10.7 h (correlation coefficient of 0.849), 7.5 h (correlation coefficient of 0.97), and 8.91 h (correlation coefficient of 0.93), respectively (average half-life of 9.06 ± 1.6 h, n = 3). Figure 6 reports curves that include the data obtained in three independent determinations of the dissociation rate in the presence of unlabeled α-NTX or α-BGT, done with different preparations of unlabeled toxins, ^{125}I-α-BGT and AcChR. Both the consistency of the dissociation rates, which are the same for all the three preparations of AcChR used, and the magnitude of the effect (in these composite curves the calculated $t_{1/2}$ is 8.75 h in the presence of α-NTX, 290 h in the presence of α-BGT) are evident.

Discussion

In this study we demonstrate that the two α-BGT binding sites on the native AcChR molecule have substantially different binding properties. One site has properties similar to those found in detergent-solubilized AcChR, and it can be measured by assays that use slow procedures to separate the bound from the free toxin. The second site binds α-BGT with much lower affinity (half-saturating concentration of approximately 60 nM) and in a reversible fashion and can be revealed by centrifugation binding assays. Triton X-100 influences the binding properties of this second site and converts it to high affinity and irreversibility. The occurrence in the native AcChR molecule of only one binding site for α-BGT that binds with high affinity and irreversibility does not conflict with the irreversible block caused by α-BGT on electroplax and muscle AcChR, because it has been demonstrated that occupancy of one site only by α-NTX (Sine & Taylor, 1980, 1981) or by the cyclic diterpenoid coral toxin lophotoxin (Culver et al., 1984) is sufficient to render the receptor nonfunctional.

The present data confirm and explain a previous study of Chang et al. (1984) in which twice as many sites for α-BGT were found when the binding assay was carried out in the presence of Lubrol. These authors proposed an "unmasking" effect of the detergent. In review of our findings, their results should be explained by the inability of their assay to reveal reversible binding.

An effect of nonionic detergent treatment on the binding properties of AcAhR for small cholinergic ligands (acetylcholine, carbamoylcholine, etc.), consisting of an increase in the affinity similar to that induced by desensitization, has been described [reviewed in Sugiyama and Changeux (1975) and Raftery et al. (1976)]. Also the interconversion of affinity states induced by agonists is lost upon dissolution of the membranes in neutral detergents or in sodium cholate (Sugiyama & Changeux, 1975), unless exogenous lipids are present (Heidmann et al., 1980). Furthermore, disruption of AcChR/membrane lipid interactions by general anesthetics such as halothane, chloroform, or diethyl ether results in an enhanced rate of conversion of the AcChR to a state or states of high affinity for carbamoylcholine (Young et al., 1978). Also, the effect of aliphatic alcohols as noncompetitive blockers of the AcChR seems to be due to a stabilization of the AcChR in a high-affinity, desensitized state similar to that stabilized by agonists, probably caused by nonspecific partitioning of the alcohols into membrane lipid or by their interactions with the receptor/lipid interface (Heidmann et al., 1983; Boyd & Cohen, 1984). It is therefore not surprising that the disruption

of the AcChR/membrane interaction induced by Triton X-100 may have a similar effect of inducing and stabilizing a high-affinity conformation.

When membrane fragments were subjected to a long incubation at 4–6 °C and occasionally in freshly purified membranes, the number of high-affinity binding sites was the same as in the presence of detergent. This could be due either to a detergent-like effect of the lysophospholipids produced by the saponifying effect of alkaline treatment or to the action of an unknown endogenous enzymatic system able to influence the affinity of α-BGT binding site(s). The finding that, on the rare occasions when newly prepared membranes had the same number of sites as in the presence of detergent, the SDS–PAGE pattern of their constituent subunits showed evidence of proteolysis argues in favor of the latter possibility. In addition, Chang et al. (1984) reported that from the same tissue they could obtain different membrane fractions, some of which displayed a different amount of binding sites for α-BGT in the presence or absence of Lubrol while other fractions did not, or they did so to a lesser extent. It is therefore conceivable that different binding states of native AchChR could exist, possibly due to endogenous proteolytic systems able to modify the properties of the low-affinity sites. Although exogenous proteolytic treatment does not cause obvious changes in AcChR function (Conti-Tronconi et al., 1982), proteases affect several structural properties of the native AcChR, including (i) conversion of dimers to monomers (Conti-Tronconi et al., 1982), (ii) modest but significant changes of the sedimentation coefficient of the monomers (Conti-Tronconi et al., 1982), (iii) subtle morphological changes of negatively stained AcChR molecules (Lindstrom et al., 1980), and (iv) induction of a higher degree of symmetry of the arrangement of AcChR molecules in membrane fragments (Bon et al., 1984).

In spite of the wealth of information on the AcChR yield by the use of snake α-neurotoxins, and α-BGT in particular, no general agreement exists on their mechanisms of interaction with the AcChR. In addition to the discrepancies that may arise from different forms of iodinated α-BGT which may bind to AcChR with different kinetics (Wang & Schmidt, 1980; Lukas et al., 1981), several of the reported disagreements may be reconciled by the results reported here. For example, Blanchard et al. (1979), who used nonproteolyzed M-AcChR and a DEAE disk assay, found only one class of noninteracting sites. They probably measured only the one irreversible site present on intact M-AcChR. These authors found for purified S-AcChR binding kinetics of α-BGT faster than M-AcChR and biphasic, consistent with the presence of two different high-affinity sites. On the other hand, Leprince et al. (1981) used assays able to detect irreversible α-BGT binding to membranes enriched in AcChR of unknown specific activity, which therefore may contain different, uncontrolled cellular and enzymatic components. The membranes they used were prepared following a protocol (Sobel et al., 1977) that does not inhibit protease activity and which is known to yield proteolyzed AcChR. These authors may therefore have detected the binding to a mixture of partially proteolyzed AcChR forms, some AcChR molecules having acquired irreversibility of the second binding site because of the protease action. In this respect, Chang et al. (1974) found heterogeneity for α-BGT binding of different AcChR-rich membrane fractions prepared by the same method (Sorbel et al., 1977). Some such fractions displayed a doubling of αHBGT binding sites after detergent treatment, while other fractions had the same number of binding sites before and after detergent treatment. In the same vein, the study of Lukas et al. (1981) was also carried out with AcChR-rich membranes of low specific activity (100 pmol of α-toxin sites/mg of protein, i.e., only 1.39% of the specific activity of pure AcChR), prepared by a method (Hazelbauer & Changeux, 1974) that does not inhibit proteases. These authors used a centrifugation assay able to detect both irreversible and reversible sites, and they found monophasic kinetics. In view of the nonpurified, proteolyzed M-AcChR they used, it is difficult to interpret their results. In favor of a major proteolytic degradation of the AcChR, able to drastically affect the AcChR binding properties, is the unusually fast dissociation of the α-BGT/AcChR complexes, which occurred over hours and which was accelerated by the presence of unlabeled α-BGT (Lukas et al., 1981). In several other studies [reviewed in Conti-Tronconi and Raftery (1986) and Maelicke (1987)], including the present one, the measured half-life of the irreversible complexes of ^{125}I-α-BGT was at least 1 order of magnitude longer and was not obviously influenced by the presence of unlabeled α-BGT.

After saturation of the irreversible site of M-AcChR and the unreacted toxin was washed away, one might expect that, upon Triton X-100 solubilization, α-BGT would bind irreversibly to the second site, which should still be free. On the other hand, it is known that after preincubation with unlabeled toxin and washing, no further irreversible binding of ^{125}I-α-BGT can be detected after Triton X-100 solubilization, a finding which has been used to demonstrate that *Torpedo* membrane fragments which spontaneously reseal form in more than 95% of cases right-side-out microsacs (Hartig & Raftery, 1979; Strader & Raftery, 1980; St. John et al., 1981). This dilemma is reconciled by the finding that occupancy by α-BGT of the irreversible site of M-AcChR inhibits the conversion of the second site to irreversibility upon Triton X-100 solubilization. The second site is, however, free and available for reversible ^{125}I-α-BGT binding, as shown in Figure 4.

Triton X-100 solubilization makes the binding properties of the two sites for α-BTX more similar, but they can still be distinguished by their slightly different kinetic properties in both *Torpedo* electric tissue (Blanchard et al., 1979) and skeletal muscle (Wang & Schmidt, 1980). The presence of bound Con A further differentiates between the two sites on S-AcChR, because it drastically affects the association rate of α-BGT to one site (Figure 5), without affecting the total bound α-BTX. Con A has been shown to inhibit 40% of the α-BTX binding to S-AcChR from *Torpedo marmorata* (Wonnacott et al., 1980). The results of that study are in excellent agreement with out data, because Wonnacott et al. (1980) used low concentrations of α-BGT and AcChR (below 1.4 nM) and a relatively short incubation (90 min). Under these circimstances only one site is fully saturated. Under similar circumstances (e.g., 90-min incubation, 2 nM α-BGT, see Figure 5) we also had only approximately 60% of the binding obtained in the absence of Con A.

The drastic effect of Con A on the association rate of α-BGT to one site raises the possibility that in *Torpedo* AcChR a sugar moiety is in close proximity to such site and that the second site either is close to a different carbohydrate chain unable to bind Con A or does not have nearby sugar domains at all. These considerations have interesting ramifications regarding both the sequence segments which form the two sites and their subunit location. If the two α-BGT sites are on the two α-subunits of a *Torpedo* AcChR oligomer, their differential sensitivity to the presence of bound Con A would be well explained by previous reports that the two *Torpedo* α-subunits are glycosylated to a diffferent extent (Raftery et al., 1983;

Lindstrom et al., 1983; Ratnam et al., 1986; Hall et al., 1983; Conti-Tronconi et al., 1984). On the other hand, in another study it was suggested that the carbohydrate parts of the two α-subunits in the AcChR monomer are identical (Nomoto et al., 1986). If this is indeed the case, it is more difficult to explain the effect of Con A on one site only if both sites are on α-subunits, unless their different microenvironments, i.e., their different flanking domains, allow Con A binding to one α-subunit only. Alternatively, one could speculate that the lower affinity site is on a different subunit, and because of its intrinsic lower affinity its binding cannot be detected after AcChR denaturation, while α-BGT binding to the α-subunit is still demonstrable (Haggerty & Froener, 1981; Gershoni et al, 1983; Wilson et al., 1984).

A direct demonstration that the two binding sites for snake α-neurotoxins are structurally different has been attained by studying the binding of derivatives of *Naja nigricollis* α toxin, spin labeled on a single amino group, by electron spin resonance (ESR) (Rousselet et al., 1984). It was found that the derivatives labeled at residues Lys^{47} and Lys^{51}, which are known to be directly involved in the interaction with AcChR, gave, upon binding to AcChR, complex ESR signals that could be interpreted as indicating the existence of two physically different binding sites.

In contrast to α-BGT, α-NTX binds in a similar, semi-irreversible fashion to both sites, in both S-AcChR and M-AcChR. The present data confirm previous studies showing that (i) α-NTX binds irreversibly to *Torpedo* AcChR with a stoichiometry of two toxin molecules to one AcChR molecule both in M-AcChR (Conti-Tronconi & Raftery, 1986) and S-AcChR (Conti-Tronconi and Raftery, unpublished results); (ii) when the binding to *Torpedo* postsynaptic membranes of either α-BGT or α-NTX was assayed by fluorescence titration methods which measure both irreversible and reversible binding, the same number of sites, two for each AcChR molecule, was demonstrated for both toxins (Neubig & Cohen, 1979); (iii) in kinetic studies on the interaction of fluorescein isothiocyanate labeled α-NTX to *Torpedo* membrane bound AcChR (Chang et al., 1984) or to solubilize *Electrophorus* AcChR (Kang & Maelicke, 1980) the association was a single, bimolecular process, with a homogeneous class of binding sites; (iv) in the muscle cell line BC3H1, α-NTX binds randomly to either of two sites, in spite of their intrinsic different binding properties for other cholinergic antagonists, like *d*-tubocurarine (Sine & Taylor, 1980, 1981; Culver et al., 1984).

The ability of α-NTX to bind in a very slowly revesible fashion to both sites, and to substitute reversibly bound α-BGT, explains the accelerated dissociation of α-BGT from its irreversible site (Figure 6). As a result of α-NTX binding to the second site, a conformational change must occur, which modifies the binding properties of the other site, and α-BGT is more quickly released. An accelerated dissociation of α-BGT/M-AcChR complexes can be induced by the α-neurotoxin from *Dendroaspis viridis* venom (α-DTX), which binds to four sites on the AcChR molecule (Conti-Tronconi & Raftery, 1986). The dissociation of complexes between certain brain AcChRs and α-BGT is accelerated by high concentrations of small cholinergic ligands (Wang et al., 1978) presumably by a similar mechanism. Small cholinergic ligands can also accelerate the dissociation of α-NTX from solubilized *Electrophorus* AcChR (Kang & Maelicke, 1980) in a manner dependent on the concentration of cholinergic ligand present (Maelicke et al., 1977; Kang & Maelicke, 1980). The dissociation rate of α-NTX/AcChR complexes can be accelerated several hundred fold above its intrinsic value by addition of high concentrations of cholinergic ligands (Kang & Maelicke, 1980). This effect may explain the well-known ability of small cholinergic ligands to competitively elute solubilized AcChR from α-NTX affinity resin, with a faster time course than would be expected from the slow dissociation rate of α-NTX/AcChR complexes. The effect of the cholinergic ligands on the dissociation of α- NTX/AcChR complexes may be due either to association with a subsite partially overlapping the α-NTX site, therefore destabilizing α-NTX binding, or to association with a site or sites not recognized by α-NTX, like the two extra sites of α-DTX.

Cholinergic antagonists exist that bind irreversibly to as many as four sites (α-DTX; Conti-Tronconi & Raftery, 1986) or as few as one site (lophotoxin; Culver et al., 1984) on the AcChR molecule. Evidence from spectroscopic studies on cholinergic agonists binding to *Torpdeo* AcChR whose two α-subunit sites were blocked by covalently attached bromoacetylcholine suggested multiple sites, possibly four, exist on the AcChR molecule and that activation and desensitization could be independent, parallel processes, triggered by ligand binding to different sites or subsites (Dunn et al., 1983). All of these suggests that a network of interacting binding sites may exist on the AcChR molecule, possibly as many as five, on the homologous domains of the AcChR, characterized by different binding properties for the different agonists and antagonists. The final functional effects of ligand binding would therefore result from the temporal summation of the single effects caused by the interplay of the differently activated sites. In this respect irreversible ligands like lophotoxin, α-BGT, α-NTX, and α-DTX, which bind to an increasing number of irreversible and reversible sites, elegantly demonstrate the spectrum of possibilities that one needs to confront when studying ligand binding to the nicotinic receptor molecule.

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