A Monoclonal Antibody Interfering with Binding and Response of the Acetylcholine Receptor*

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Employing a monoclonal antibody raised against the receptor protein, we have probed the mechanism of ligand interaction of the nicotinic acetylcholine receptor from Torpedo marmorata.

Antibody WF6 specifically binds to α-subunits of the receptor with a stoichiometry of one molecule per receptor monomer. At saturating concentrations, WF6 blocks half of the binding sites for acetylcholine, all of the binding sites for α-neurotoxins, and none of the binding sites for representative cholinergic antagonists (with the exception of α-toxins) at the receptor. In the presence of saturating concentrations of antibody WF6, acetylcholine (or its agonists) cannot induce Tl⁺ influx into Torpedo membrane vesicles. Rapid oversaturation of the receptor by agonist also cannot overcome this blockade of channel gating.

The observed competition patterns of WF6 and representative cholinergic ligands with the receptor are evidence for separate binding sites for groups of ligands and for a network of allosterically linked effector regions at the receptor. The blockade by saturating concentrations of WF6 of the agonist-induced channel gating supports the conclusion that two molecules of agonist are required to activate the receptor-integral ion channel.

The nicotinic acetylcholine receptor (AChR)† from fish electric tissue is an integral signal transducer (1); it contains in its protein moiety binding sites for acetylcholine and its agonists and antagonists (receptor function), the ligand-gated cation channel (response function), and several types of modulator sites (modulator functions). Despite the fact that the AChR is the best studied of all neuroreceptors, a full understanding of its molecular mechanism of action has not yet been achieved. This may be exemplified by the existence of several basically different models for the receptor's activation-inactivation cycle (2-4).

As a new approach to probe into the receptor's mechanism of action, we have begun to investigate the involvement of specific surface domains in receptor function. The particular domains are characterized by the binding of monoclonal antibodies raised against the AChR (5). Employing these novel ligands in functional studies of the receptor, i.e. ligand binding and kinetic, ion flux, and physiological studies, we can show that certain groups of receptor ligands bind to separate sites and compete by allosteric mechanisms for receptor binding. Our studies support the conclusion (6-10) that two molecules of acetylcholine (or agonist) are required to activate the receptor-integral ion channel. As a general conclusion, there apparently exists a network of coupled domains at the receptor surface which by its interplay controls receptor function.

MATERIALS AND METHODS

The cholinergic ligands and local anesthetics employed were obtained from Sigma; peroxidase-labeled second antibody was from Dakopatts, Hamburg, West Germany. All gel electrophoresis and blotting materials were from Bio-Rad. TlNO₃ was from Merck; immunoprecipitin (formaldehyde-fixed Staphylococcus aureus cells) was from GIBCO; [³H]acetylcholine (2.7 Ci/mmol), [¹²⁵I]-a-bungarotoxin (254 Ci/mmol), [¹²⁵I]-labeled anti-mouse IgG (11 μCi/μg), and [¹²⁵I]-protein A (33 μCi/μg) were from Ameraizm Corp.; and gold-labeled protein A (12-nm gold size) was from Janssen Pharmaceutical, Beense, Belgium. Endoglugucosidase H was from Miles Laboratories Inc.; and saponin was from Sigma. All other chemicals and biochemicals were obtained from standard sources as previously described (6, 11).

The procedures for the preparation and tritiation of antibody WF6 were described previously (5). Antibody concentrations were determined with the extinction coefficient (in PBS) of E₅₆₅ = 1.4. Torpedo membrane fragments were prepared as described in Ref. 11. Acetylcholine receptor from Torpedo marmorata was solubilized and purified according to Ruchel et al. (12). Receptor subunits were prepared by preparative SDS gel electrophoresis according to Ref. 13. α-Neurotoxin from Naja naja siamensis was purified and tittated as described by Maelicke et al. (14). Membrane preparations from head ganglionic tissue of Locusta migratoria (15) were a gift from Dr. H. Breer, Zoologisches Institut, Universität Osnabrück (Osnabrück, West Germany).

Electron Micrographs

Torpedo membrane fragments in PBS were successively labeled with WF6 and gold-labeled protein A. Incubations were each carried out for 6-10 h at 4 °C with 3-fold washing with PBS after each incubation step. In the final wash, 10-fold dilute PBS was employed, and the membrane fragments were immediately quick-frozen (16). Specimens prepared in this way were freeze-substituted, sectioned, and electron-micrographed as described (17). (These experiments were performed at the Marine Biological Laboratory (Woods Hole, MA) in collaboration with Dr. T. S. Reese (18) and by D. Veltel, Institute of Medical Cytobiology, University of Münster (Münster, West Germany).)

Sucrose Gradient Centrifugation

To determine the stoichiometry of receptor-antibody binding, solubilized receptor (∼5 × 10⁻⁸ M) in PBS/Tween (0.05% Tween 20)
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was incubated overnight with either [3H]α-cobratoxin (5 × 10^-6 M) or [3H]-labeled WF6 (1 × 10^-6 and 1 × 10^-7 M, respectively) and layered on top of a 5%-20% (w/v) sucrose gradient in PBS/Tween. After centrifugation with a VTI-65 rotor at 220,000 × g for 45 min, fractions of 100 μL were collected, and their content of tritiated material was determined.

**Immunoblot Procedures**

Torpedo membrane fragments were electrophoresed according to Laemmli (19) on 10% SDS gels (12 × 16 × 0.1 cm) at 8 mA constant current (10-50 μg of protein/slot). If desired, the membrane fragments were pretreated with endoglucanase H as described (20). Staining of protein bands was accomplished with Coomasie Blue and the remaining slots were blotted (21) for 2 h at 50 V onto nitrocellulose paper (BA85, 0.45 μm, Schleicher & Schüll) in transfer buffer (154 mM glycine, 20 mM Tris, 20% methanol). Again, one slot each of protein standards and membrane fragments was stained for comparison. The unreacted sites of the other slots were blocked by overnight incubation with BSA (10 mg/ml in blotting buffer: 0.9% NaCl, 10 mM Tris/Cl, pH 7.4). The paper was then incubated for 5-15 h with WF6 (5 μg/ml) and 10 mg/ml BSA in PBS, washed (five times for 5 min each), further incubated with PBS/Tween and two times with water, and finally submitted to autoradiography employing a Fuji x-ray (Medical) film (exposure time: 7-20 days). Incubation of blotted receptor with [3H]-α-bungarotoxin (1 × 10^6 cpm/ml) was performed overnight followed by washing and autoradiography as described above. Alternatively, the receptor-antibody complex was detected by employing peroxidase-labeled second antibody as described (18).

**WF6 Binding Assays**

**Membrane-bound Receptor—Torpedo** membrane fragments in assay buffer (100 mM NaCl, 4 mM CaCl2, 2 mM MgCl2, 50 μg/ml BSA, 20 mM Hepes, pH 7.4) were incubated for the periods of time indicated in the figure legends at 4 °C with given concentrations of [3H]-labeled WF6 (80 Ci/mmol). Separation of bound and free ligand was achieved by an air-dried centrifuge according to established procedures (11). The effect of acetylcholine on WF6 binding was determined by simultaneous incubation of acetylcholine and [3H]-labeled WF6 with esterase-blocked (see Ref. 11) membrane fragments.

**Solubilized Receptor—Affinity-purified** receptor in standard buffer (125 mM NaCl, 25 mM Tris/Cl, pH 7.4, 1% (w/v) Tween 80) was incubated for the indicated periods of time at 4 °C with varying concentrations of WF6. Receptor-antibody complexes were immunoprecipitated by addition of 0.15 volume of immunoprecipitin. 3 h after precipitation, the remaining AChR in the supernatant (i.e. functional receptor) was determined by the absorbance at 490 nm read in a Gilford automatic Mikro-ELISA Analyzer PR50.

**Initial Rate of Binding—Torpedo** membrane fragments (1 × 10^-9 M in AChR) and [3H]-labeled WF6 (1 × 10^-10 M, 80 Ci/mmol) in assay buffer were incubated in the absence and presence of acetylcholine and carbamoylcholine, respectively. Aliquots were then incubated without further incubation for 15 min (ligands) or 2 h (toxin) followed by overnight incubation with [3H]-α-cobratoxin (5 × 10^-6 to 5 × 10^-5 M). Bound and free-α-toxins were assayed by the rapid centrifugation assay as described (11).

**Toxin Binding Assay**

Torpedo membrane fragments (~2 × 10^10 M in AChR) were pre-equilibrated with WF6 (5 × 10^-10 M) followed by overnight incubation with [3H]-α-cobratoxin (5 × 10^-6 to 5 × 10^-5 M). Bound and free-α-toxins were assayed by the rapid centrifugation assay as described (11).

**Competition Binding Assays**

By ELISA—For competition ELISAs, Gilford Cuvette Paks were coated overnight at 4 °C with solubilized AChR (~10^-10 M) or Torpedo membrane fragments (~5 × 10^-10 M). Excess antigen was removed by washing twice with PBS/Tween. Ligand solutions or PBS/Tween (control) was then added, and the plates were incubated at room temperature for 15 min (ligands) or 2 h (toxin) followed by the addition of antibody (initial concentration: 0.5 μg/ml in PBS) and further incubation for 5 h at 37 °C. After three washes with PBS/Tween, peroxidase-labeled anti-mouse IgG (11,000 in PBS/Tween) was added and incubated for 1 h at 37 °C. After washing three times with PBS/Tween and two times with water, the substrate solution (15 mM o-phenylenediamine, 1.5% (v/v) H2O2, 0.1 M citric acid, pH 5.0) was added; and, after 20 min at 37 °C, the reaction was stopped with 0.5 volume of 4.5 M H2SO4. The extent of enzymatic reaction was determined by the absorbance at 490 nm read in a Gilford automatic Mikro-ELISA Analyzer PR50. ACh Binding Assay

These were performed as previously described (11). The quantitative analysis of data was performed by employing a two-population model (Scheme 1) assuming two classes of positive cooperatively interacting sites for ACh (11) and one class of sites for WF6 at the receptor (the related computer programs were developed by Dr. E. K. Wolfs of our laboratory).

**RESULTS**

Antibody WF6 is a mouse anti-Torpedo AChR antibody produced by the hybridoma cell line XR6-G10-B3. It was obtained by recloning, after a large number of passages,
hybridoma cells originating from clone XR6-G10 (5).

**Immunological Characterization of WFS**—As its parent clone, WFS belongs to the IgG subclass 2a and binds to both membrane-bound and purified receptors from *T. marmorata* and, with much lower affinity, also to purified receptor from *Electrophorus* electric organ and membrane preparations from rat myotubes. It does not cross-react with *Locusta* head ganglionic tissue (24). WFS binds selectively to the acetylcholine receptor as compared to a variety of water-soluble and membrane-bound proteins including acetylcholine esterase from *Torpedo* and *Electrophorus*.

WFS binds to the extracellular region of the AChR—Fig. 1 is an electron micrograph of rapidly frozen and freeze-substituted *Torpedo* membrane fragments labeled successively with WFS and gold-labeled anti-mouse IgG. The figure depicts a mixture of cross-fractured vesicles from the innervated and noninnervated sides of *Torpedo* electroplaques. One of the AChR-carrying vesicles is not cross-fractured but lies just beneath the fracture plane. The observed labeling pattern is specific for AChR-carrying vesicles and requires the presence of the first antibody (WFS). In the many samples analyzed in this way, we observed only outside labeling of the vesicles. In particular, the labeling pattern was not changed in the presence of saponin, showing again that no inside-out vesicles were present in the preparation.

**Stoichiometry and Subunit Specificity of WFS Binding**—Several methods were employed to determine the number of WFS-binding sites per receptor monomer. Of these, the sucrose gradient centrifugation method (26, 27) is the only one that is independent of the specific activity of labeled (e.g., tritiated) or unlabeled WFS. As is representative shown in Fig. 2, mixtures of antibody and receptor sedimented either as mixtures of free WFS and complexes of one WFS and one AChR molecule (in the presence of excess WFS) or as complexes of one WFS and two AChR molecules (in the presence of excess AChR). Under no conditions did we observe a stoichiometry of two antibody molecules per one receptor molecule. Thus, the stoichiometry of WFS binding is one per receptor monomer. This agrees with the stoichiometry obtained from the direct binding and acetylcholine competition experiments shown below.

As shown by the immunoblotting experiment of Fig. 3, WFS specifically binds to α-subunits of the AChR. As two α-subunits exist per receptor monomer, the above observed stoichiometry of WFS binding could have been due to sterical protection of the second α-subunit after WFS has bound to the first or to inherent structural differences in the two α-subunits. Different degrees of N-glycosylation have been proposed for the latter (28). Immunoblotting experiments before and after treatment of the receptor with endoglycosidase H show, however, that the binding of WFS is not affected by the degree of N-glycosylation (Fig. 3). Hence, it is probably due to sterical hindrance that only one α-subunit per receptor monomer can serve as binding site for antibody WFS.

**Affinity and Kinetics of Binding of WFS**—Preliminary experiments (5) already indicated that WFS binds with high affinity and very small dissociation rate constant to the receptor. These properties call for extremely long incubation times in equilibrium binding experiments (14) and therefore limit the reliability of the related data. Thus, the experiments of Fig. 4 only permit the setting of a lower limit for the affinity of binding of WFS to the receptor. The *Kd* value of the interaction is of the order of 1 × 10^{-11} M (or less), and the apparent lower affinities determined after incubation times of less than 1 week probably are due to incomplete equilibrium. *Kd* values of the order of 10^{-10} M were also obtained for the interaction of WFS with solubilized, affinity-purified receptor (not shown).

Dissociation of preformed WFS-receptor complexes was determined by dilution with assay buffer containing excess of unlabeled WFS or α-bungarotoxin (Fig. 5). Both experiments yielded similar results. From the dominant component of the semilogarithmic plot, a dissociation rate constant of the order of *k_{-1} = 5 × 10^{-5} s^{-1}* was obtained. As only little more than one half-life of the dissociation kinetics was monitored, the kinetic pattern and rate constant only provide preliminary information on the nature of this interaction.

The association kinetics of WFS and membrane-bound *Torpedo* receptor were determined by initial rate measurements at different concentrations of receptor and antibody. The obtained association rate constants were of the order of 10^3-10^5 M^{-1} s^{-1}. Taken together, the equilibrium binding and kinetic data on the interaction of WFS with *Torpedo* receptor

![Image](image-url)
characterize antibody WF6 as a ligand matching the snake α-neurotoxins in both affinity and kinetic properties (14).

**Competition of WF6 and Cholinergic Ligands for Receptor Binding**—To obtain a qualitative overview as to which ligands compete with antibodies for receptor binding, simple ELISA dilution experiments can be employed (5). By using identical experimental conditions (the same receptor, antibody, and ligand concentrations), this method already permits the establishment of an order of competition (from strong to no competition at all). Fig. 6 is a representative example of experiments of this kind. Taken together, the ELISA experiments yielded the following order of competition: α-cobra toxin > acetylcholine, NBD-5-acylcholine >> carbamoylcholine, suberyldicholine > thiocarbamoylcholine, acetylethiocholine > nicotine > choline > decamethonium, hexamethonium, galamine. No competition (within the limits of experimental error) was observed with tubocurarine and the local anesthetics dibucaine and quinacrine. Thus, agonists (with the exception of decamethonium) and α-neurotoxins competed with WF6 for receptor binding in accordance with their established order of affinities (22). In contrast, low molecular weight ligands known to act as antagonists or local anesthetics at the Torpedo receptor did not show competition with WF6 for receptor binding.
These findings agree with and expand previous observations from our laboratory (5). They suggest the possibility of separate sites for groups of cholinergic ligands at the receptor, with all its consequences for the basic mechanisms of receptor function. The following experiments were undertaken to further probe into this direction.

Competition between WF6 and α-Cobratoxin for Receptor Binding—In view of the stoichiometry of WF6 binding to the receptor (one WF6 molecule per receptor monomer), it was interesting to test whether WF6 competed with only one or both toxin molecules known to bind per receptor monomer (1). Fig. 7 exemplifies the result of two sets of experiments performed for this purpose. As shown in Fig. 7A, antibody WF6 can completely prevent binding of α-cobratoxin to the receptor. This agrees with the notion of one WF6 molecule binding mutually exclusively with two toxin molecules to the receptor. In the second type of experiments (Fig. 7B), we determined the affinity of toxin binding in the presence of antibody WF6. According to these experiments, WF6 reduces the number of binding sites available for toxin at the receptor without any effect on the binding affinity of the toxin. Together with our previous finding that neurotoxin can completely prevent binding of WF6 to the receptor, these results establish that one WF6 molecule binds mutually exclusively with two neurotoxin molecules per receptor monomer. Again, as for the binding stoichiometry of one WF6 molecule per two α-subunits, the toxin competition data could simply be the result of sterical protection of both binding sites by the single (large) antibody molecule bound (nonspecifically) to one of the two α-subunits.

Competition between WF6 and Acetylcholine for Receptor Binding—The following experiments were performed to establish the mode of competition between WF6 and the natural transmitter for receptor binding. (i) Scatchard plots of the binding of tritiated WF6 in the absence and presence of constant concentrations of acetylcholine (Fig. 8A) established that acetylcholine reduces the number of available WF6 sites without any effect on the binding affinity of WF6. (ii) Scatchard plots of the binding of [3H]acetylcholine in the absence and presence of constant concentrations of WF6 to the receptor established that, at saturating concentrations, WF6 blocks only one-half of the acetylcholine-binding sites at the receptor (Fig. 8B). Under conditions of saturating blockade by WF6, the class of cooperatively induced high affinity sites for acetylcholine disappears. In contrast, the class of low affinity sites for acetylcholine is not affected by the presence of WF6. In summary, there exists mutually exclusive binding of one WF6 molecule with one acetylcholine molecule per receptor monomer and independent (noncompetitive) binding of a second acetylcholine molecule.

Kinetics of Competition of WF6 and Acetylcholine (or Carbachol)—Since radioactively labeled agonists of acetylcholine are commercially not readily available, we employed a kinetic competition assay to extend the qualitative data of the competition ELISA (Fig. 6) to one more agonist of acetylcholine. As shown in Fig. 9, the initial rate constant of WF6 binding to the receptor (~1 x 10^6 M^-1 s^-1) is reduced in proportion to increasing concentrations of acetylcholine and carbachol, respectively. Approximately 10–50 times more carbachol than acetylcholine is required

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**FIG. 6. Ligand competition ELISAs with Torpedo membrane fragments and WF6.** The serial dilutions of WF6 were performed as described under "Materials and Methods." Dilutions were in the absence of ligand (X), in the presence of acetylcholine (V), carbachol (O), decamethonium (A), hexamethonium (Δ), quinacrine (C), and tubocurarine (O) at initially 10^-5 M, and in the presence of initially 10^-6 M α-cobratoxin (V). The data points referring to WF6 dilution in the presence of 10^-3 M tubocurarine and quinacrine were considered indistinguishable from the control data (X); those in the presence of hexamethonium and decamethonium, although constantly differing from the control curve, were just within the limits of experimental error.

**FIG. 7. Competition binding of α-cobratoxin and WF6 to membrane-bound receptor from T. marmorata.** A. Torpedo membrane fragments (1 x 10^-8 M in AChR) were incubated overnight at 4°C with the given concentrations of WF6 followed by incubation overnight with [3H]α-cobratoxin (1 x 10^-7 M) as described under "Materials and Methods." Free and bound α-toxins were assayed by a rapid centrifugation assay (11). B. Torpedo membrane fragments (2.6 x 10^-9 M in AChR) were incubated for 6 days at 4°C without (○) and with (×) 5 x 10^-9 M WF6 followed by incubation for 1 day with [3H]α-cobratoxin. The following Kd values and R0 values in the absence and presence of WF6 were obtained: Kd = 2.5 x 10^-7 and 2.9 x 10^-8 M, respectively; R0 = 2.8 x 10^-9 and 7.6 x 10^-10 M, respectively.
denotes the fraction of antibody-occupied receptor and was calculated
values agree with previous results
value is close to
performed assuming two classes of positive cooperatively interacting
according to the equation provided under “Materials and Methods.”
acetylcholine in the mixed complexes with WF6 and the receptor. Its
Kfj1
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the rapid centrifugation assay described under “Materials and Meth-
ods.” The Scatchard representations of the three sets of binding data
concentrations of WF6 (see the following table) followed by incuba-
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= 5 \times 10^{-11} \text{M}
B, Torpedo membrane fragments
were incubated for 16 h at 4 \text{°C} with varying concentrations of WF6 (see the following table) followed by incubation with the given concentrations of [3H]acetylcholine for 15 min. Bound and free acetylcholine were separated by rapid centrifugation (see “Materials and Methods”). The quantitative analysis of data was performed assuming two classes of positive cooperatively interacting sites for acetylcholine (11) and a single class of sites for WF6. For details concerning the employed model and algorithm, see “Materials and Methods” and Ref. 11. The following data were obtained.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>cWF6</th>
<th>cACHR</th>
<th>( \alpha )</th>
<th>( K_D1 )</th>
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\( K_D1 \) and \( K_D3 \) are the equilibrium dissociation constants for the two classes of acetylcholine-binding sites in the absence of WF6. Their values agree with previous results (11). \( K_D2 \) is the \( K_D \) value for acetylcholine in the mixed complexes with WF6 and the receptor. Its value is close to \( K_D1 \), i.e. the low affinity site for acetylcholine. \( \alpha \) denotes the fraction of antibody-occupied receptor and was calculated according to the equation provided under “Materials and Methods.”

to reduce the initial rate of WF6 binding to one-half of its value in the absence of competing agonist. This agrees with the established difference in binding affinity of these two agonists (1, 22).

**Cross-competition between Groups of Cholinergic Ligands**—To further assess the possibility of separate binding sites for groups of cholinergic ligands (Fig. 6), we have tested whether and to what extent a third ligand interferes with the competition of WF6 and another ligand for receptor binding. For this purpose, we have grouped the ligands as follows: Group I, acetylcholine and its agonists (with the exception of decamethonium): mutually exclusive binding with WF6 to one-half of their sites, independent binding to the other half of their sites; Group Ia, \( \alpha \)-neurotoxins: mutually exclusive binding with WF6 to all of their sites but different binding stoichiometry (2:1) than WF6 (1:1) with the receptor; Group II, low molecular weight antagonists: no binding competition with WF6. (Based on our assays, decamethonium had to be placed in Group II which agrees with the established in vitro properties of *Torpedo* membrane vesicles (29, 30).)

The following experiments exposed the cross-competition properties most clearly. (i) As shown in Fig. 10A, the association rate of WF6 to the receptor was reduced in the presence of high concentrations of acetylcholine (or NBD-5-acylcho-
line or succinyldicholine), whereas the same concentration of hexamethonium (or tubocurarine) did not affect the WF6 association kinetics at all. However, if hexamethonium was applied together with acetylcholine in the competition kinetics, the blockade of WF6 binding by acetylcholine was reduced and, at sufficient excess of hexamethonium over acetylcho-
line, completely disappeared (Fig. 10B). Obviously, we observed competition between hexamethonium and acetylcho-
line for receptor binding which reduced (and at saturating concentrations completely eliminated) the competing effect of acetylcholine on WF6 binding. The protecting effect of hexamethonium on acetylcholine-WF6 competition has been observed under a variety of experimental conditions including cross-competition ELISAs (Fig. 10C). Furthermore, the effect is not limited to the pair acetylcholine-hexamethonium but has been observed with all pairs of Group I-Group II ligands tested so far.
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Ion Channel Blockade by WF6—The effects of WF6 binding on receptor channel activation were tested by following the kinetics of agonist-induced T1+ influx into Torpedo membrane vesicles (22, 31, 32) in the absence and presence of WF6. As shown in Fig. 11, saturating concentrations of WF6 while blocking only one-half of the agonist-binding sites (Fig. 8) completely prevented any carbamoylcholine-induced activation of the channel. Even at 600 μM carbamoylcholine, the WF6-induced blockade of channel activation was not removed. These experiments show that the receptor-integral ion channel cannot be activated when WF6 is bound to the receptor. This suggests the requirement of two bound acetylcholine molecules for ion channel activation. Alternatively, it could be argued, however, that WF6 locks the receptor in the “closed” conformation so that a gating stoichiometry cannot be deduced from our data.

DISCUSSION

Antibody WF6 is a novel type of ligand of the nicotinic acetylcholine receptor from Torpedo: similar to α-neurotoxins, it binds with very high affinity and small dissociation rate constant to the receptor, and it also acts as competitive antagonist of acetylcholine. The antagonistic action, however, is not produced by mutually exclusive binding with agonists to all of their sites but by competition with only one of the two agonist sites per receptor monomer (Fig. 8). Furthermore, antibody WF6 does not significantly compete at equilibrium.

**Fig. 10.** Effect of hexamethonium on the competition of acetylcholine and WF6 for binding to Torpedo membrane fragments. Concentrations of AChR and 3H-labeled WF6 were ~1 × 10^{-8} and 5 × 10^{-15} M, respectively. For all other experimental details, see “Materials and Methods.” A, kinetics of WF6 binding in the absence (X) and presence (C) of 10^{-4} M acetylcholine. A similar effect as with acetylcholine was observed with NBD-5-acylcholine (A) and succinylcholine (C), both at 10^{-4} M. The same concentration of hexamethonium (D) did not affect the association kinetics; the strongest competition was observed with α-cobratoxin (O, 10^{-8} M). B, kinetic competition (after 3 min) of acetylcholine (1 × 10^{-4} M) and WF6 (5 × 10^{-10} M) in the presence of varying concentrations of hexamethonium. The dashed lines indicate the levels of WF6 binding in the presence of only hexamethonium (upper line) and in the presence of only acetylcholine (lower line). C, serial dilution ELISAs of WF6 (initial concentration: 10^{-9} M) in the absence (X) and presence of acetylcholine (10^{-4} M) and the following concentrations of hexamethonium: O, no hexamethonium; A, 10^{-4} M; C, 10^{-5} M; and I, 10^{-6} M. In the control experiment in the absence of acetylcholine (C), 10^{-6} M hexamethonium had, if any, a very weak competitive effect on WF6 binding.

**Fig. 11.** Carbamoylcholine-induced T1+ influx into Torpedo membrane vesicles in the absence and presence of WF6. A: lower trace, T1+ induced fluorescence quenching resulting from T1+ influx into Torpedo membrane vesicles (2 × 10^{-7} M) after rapid mixing with 100 μM carbamoylcholine; upper trace, same experiment with Torpedo membrane fragments pretreated for 6 h with 1 μM WF6. The upper trace coincided with the control (influx in the absence of carbamoylcholine). For experimental details, see “Materials and Methods.” B, apparent first-order rate constants (k_{app} (s^{-1})) of carbamoylcholine-activated T1+ influx into Torpedo membrane vesicles preincubated without (O) and with (C) 1 μM WF6. For data analysis see Refs. 22 and 23.
with representative cholinergic antagonists other than \( \alpha \)-neurotoxins (Figs. 6 and 10).

A simple explanation for the observed stoichiometries of binding of WF6, \( \alpha \)-neurotoxin, and acetylcholine to the receptor is provided by the relative size of these ligands: the antibody molecule may be too large in size to permit simultaneous binding of two WF6 molecules to the two \( \alpha \)-subunits of receptor monomers. Thus, although the \( \alpha \)-subunits may well be identical in structure with respect to their epitope for WF6, binding of a second WF6 molecule may be sterically hindered by the presence of the first. Likewise, WF6, although bound to only one of the \( \alpha \)-subunits, may sterically hinder binding of \( \alpha \)-neurotoxin to both \( \alpha \)-subunits. In contrast with the much smaller acetylcholine, WF6 may only compete at the particular \( \alpha \)-subunit to which it is specifically attached. The much smaller \( \alpha \)-neurotoxin, WF6 may only compete at the receptor. We show (Fig. 10) that Group I and I1 ligands continue to compete with each other in the presence of WF6, binding of a second WF6 molecule may be sterically hindered by the presence of the first. Likewise, WF6, although bound only to Group I, but not Group II, ligands compete with WF6 for receptor binding. Accordingly, at least one of the three ligands employed must bind to sites separate from those for the other ligands. Keeping to the classical view that the antibody as an allosterically coupled agonist site.

In conclusion, monoclonal antibody WF6 is a novel marker molecule and ligand of the AChR. By its unusual binding properties, it permits new insights into the receptor's mechanism of action. By the results obtained so far, it is suggested that (i) sites for mutual exclusively binding ligands are not necessarily identical but may rather be separate sites which are allosterically coupled and (ii) receptor channel activation probably requires the simultaneous occupancy of two agonist sites per receptor monomer. Our study suggests that monoclonal antibodies with function-blocking activity may be useful tools in further unraveling the receptor's mechanism of action.

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REFERENCES