Interaction of Nicotinic Acetylcholine Receptor with Two Monoclonal Antibodies Recognizing Different Epitopes†

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ABSTRACT: The interactions of nicotinic acetylcholine receptor (nAChR) with two monoclonal antibodies (mAb370A and mAb371A) which block the agonist-induced ion flux into nicotinic acetylcholine receptor vesicles [Donnelly, D., Mihovilovic, M., Gonzalez-Ros, J. M., Ferragut, J. A., Richman, D., & Martinez-Carrion, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7999] have been studied by a combination of immunochemical and spectroscopic techniques. Both mAbs are specific for the α-subunit of the receptor, but they recognize different epitopes. We have detected specific binding of the mAb370A to a synthetic peptide corresponding to residues α187–205, a sequence known to contain the α-bungarotoxin binding site. By contrast, mAb371A seems to recognize an epitope which is largely silent after proteolytic digestion of the subunit. Binding of mAb370A to the receptor is inhibited by cholinergic agonists and α-neurotoxins but not by competitive antagonists or local anesthetics. By contrast, none of these ligands interferes with binding of mAb371A. The spectroscopic properties of the fluorescent probe ethidium have been used to investigate the effect of the mAbs on the interaction of the agonist carbamylcholine with nAChR in membranes. mAb370A, but not mAb371A, blocks both the agonist-induced increase in the fluorescence intensity of receptor-bound ethidium and the agonist-induced increase in the polarization value of the probe. In addition, measurements of ethidium binding followed by stopped-flow techniques showed that mAb370A, but not mAb371A, blocked the agonist-induced association of the probe to nAChR membranes. Therefore, mAb370A acts as a specific nAChR ligand which binds to an epitope which contains parts of the α-subunit sequence between residues 187–205 and competes with cholinergic ligands blocking the receptor ion channel opening response. By contrast, mAb371A acts through an, as yet, uncharacterized long-distance, noncompetitive mechanism, the latter being consistent with a conformational change of the nAChR induced by mAb371A, as reported for interactions of neuraminidase and antibodies [Colman, P. M., Laver, W. G., Varghese, J. N., Baker, A. T., Tulloch, P. A., Air, G. M., & Webster, R. G. (1987) *Nature 326*, 358–363]. Such conformational changes could shift the close–open channel equilibrium toward a closed conformation or can prevent protein movements necessary for ion channel opening.

Monoclonal antibodies (mAbs)[1] are powerful agents for the analysis of protein surfaces. Recent advances have indicated that in some proteins the interaction between a protein and antibodies can lead to significant alterations of the host protein structure (Colman et al., 1987). In the nicotinic acetylcholine receptor (nAChR), the analysis of such antibody–protein interactions has an impact beyond basic molecular understanding of the process as antibodies against nAChR are naturally produced in the debilitating, human disease *Myasthenia gravis* (Lindstrom et al., 1988). For the nAChR, there is an extensive array of mAbs produced against various regions, yet the effect of most of these antibodies in receptor function is unknown, and the consequences of such interactions on receptor structure are largely unexplored.

The nAChR is a membrane glycoprotein localized in the postsynaptic side of the mammalian neuromuscular junction and is also found in high concentrations in the electric tissue of certain fishes (Conti-Tronconi & Raftery, 1982; Changeux et al., 1984). The nAChR from *Torpedo californica* is a pentameric glycoprotein composed of four homologous subunits in the stoichiometry $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980). The genes encoding each subunit have been cloned, and the amino acid sequences have been inferred from cDNA sequences (Noda et al., 1982, 1983; Claudio et al., 1983; Devillers-Thiery et al., 1983). Analysis of the hydrophobicity profiles of the primary sequences has led to several models for the transmembrane topography of the receptor subunits (Noda et al., 1982; Claudio et al., 1983; Guy, 1983; Devillers-Thiery et al., 1983; Finer-Moore & Stroud, 1984).

The nAChR is also a ligand-gated ion channel; binding of ACh to ligand sites located on each α-subunit induces a transient opening of the cation channel, leading to a depolarization of the postsynaptic membrane. The prolonged presence of activating ligands induces desensitization of the receptor, which is characterized by a reversible inactivation of the ion channel and a stabilization of the high-affinity state of the receptor for agonists. There exist several models describing the cycle of activation–inactivation of the receptor

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[1] Abbreviations: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; mAb, monoclonal antibody; BGTx, α-bungarotoxin; CBTx, α-cobratoxin; CbCh, carbamylcholine; IMIP, imipramine; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; MG, *Myasthenia gravis*.

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(Raftery et al., 1983; Changeux et al., 1984; Hess et al., 1984).

Monoclonal anti-nAChR antibodies are potential probes for the study of nAChR structure–function relationships (Gomes et al., 1979; Tzartos & Lindstrom, 1980; Watters & Maelicke, 1983). The binding specificities of some of them have been determined (Gullick et al., 1981; Conti-Tronconi et al., 1981; Ratnam et al., 1986a) and used to test the current models for the transmembrane topology of the receptor (Ratnam et al., 1986b). mAbs raised against synthetic peptides corresponding to segments of the nAChR primary sequence have also been used to map various domains on the receptor molecule, such as the α-bungarotoxin binding site (Wilson et al., 1985; Mulac-Jericevic & Atassi, 1986; Ratnam et al., 1986a; Neumann et al., 1986a,b; Ralston et al., 1987) and the main immunogenic region (Barkas et al., 1987; Tzartos et al., 1988); nevertheless, there are still gaps of information identifying the sites of mAb binding and the assignments of the epitopes corresponding to those mAbs.

It is possible to investigate the effect of monoclonal antibodies on nAChR, particularly through the use of continuous assays for the interaction of ligands with nAChR. Such ligand interactions are reasonably well characterized for nAChR in membranes through the use of different experimental approaches, including detection of changes in the intrinsic fluorescence of the receptor upon binding of ligands (Bonner et al., 1976) or of the fluorescence of the ligands upon binding to the receptor (Martinez-Carrion & Raftery, 1973; Bode et al., 1979; Heidmann & Changeux, 1979). Additional information can be obtained by using extrinsic fluorescent probes like quinacrine (Grunhagen & Changeux, 1976), propidium (Sator et al., 1977), and ethidium (Quast et al., 1979; Schimerlik et al., 1979a,b; Herz et al., 1987), and the kinetics of agonist-mediated cation transport across membrane vesicles containing nAChR can be studied in the millisecond time scale by stopped-flow spectroscopy (Moore & Raftery, 1980; Donnelly et al., 1984; Karpen & Hess, 1986; Fels et al., 1988). Therefore, some of these methods can be used to follow the consequences of monoclonal binding to interfere with selected nAChR function on the above-mentioned procedures. Other aspects of nAChR function can be studied through the use of compounds which are neither agonists nor competitive antagonists and block the nAChR function without preventing the binding of agonists. Such compounds are known as noncompetitive antagonists and include aromatic amines (Boyd & Cohen, 1984), local anesthetics, imipramine (Shaker et al., 1981), histrionicotoxin, and phencyclidine (Eldefrawi et al., 1980; Oswald et al., 1983).

Studies using some of the above-described methodologies have been limited as to the effect of some mAbs on the mechanism of binding of different cholinergic ligands and the ion channel opening (Mihovilovic & Martinez-Carrion, 1979; Donnelly et al., 1984; Mihovilovic & Richman, 1984; Fels et al., 1986). Many mAbs have been selected for their specificities against epitopes that when blocked interfere with cholinergic ligand binding. Yet, we are particularly interested in analyzing also those directed against epitopes that do not overlap cholinergic binding sites. The latter case is important since we have already (Donnelly et al., 1984) identified a mAb that does not interfere with BGTx or CbCh binding but blocked ion channel opening.

Thus, we now report the study of the interaction of two mAbs, one presumably directed against a BGTx binding region (mAb370A) and another against a region that does not overlap the neurotoxin binding surface (mAb371A). We identify some of the protein sequence cross-reacting with the mAb against the toxin binding region and use these mAbs to study the effect of their binding on the properties of nAchR membranes.

Experimental Procedures

Materials. Frozen electroplax tissue or live *Torpedo californica* were purchased from Pacific Biomarine, Venice, CA. Electroplax tissue was stored at −70 °C until needed. α-Bungarotoxin was purified from *Bungarus multicintus* venom (Miami Serpentarium Laboratories, Salt Lake City, UT) and ^{14}C labeled by the reductive methylation method (Mebs et al., 1972; Calvo-Fernandez & Martinez-Carrion, 1981; Garcia-Borron et al., 1987). ^{125}I-BGTx and ^{125}I protein A were from New England Nuclear. α-Cobratoxin was isolated from *Naja naja siamensis* venom (Karlson et al., 1971) obtained from Miami Serpentarium Laboratories. Ethidium bromide was purchased from Molecular Probes, Eugene, OR. The cholinergic ligands and local anesthetics were from Aldrich or Sigma.

Rat IgG and rabbit anti-rat IgG were from ICN Immunobiologicals; peroxidase-conjugated goat anti-rat IgG was from Cooper Biomedical, Malver, PA, and the peroxidase substrate kit from Bio-Rad Laboratories, Richmond, CA.

nAChR-enriched membranes were prepared as described (Garcia-Borron et al., 1987), and solubilization of nAChR was achieved with 1% sodium cholate. Protein concentrations were determined by the method of Lowry et al. (1951) and specific BGTx binding activities by the DEAE filter disk assay (Schmidt & Raftery, 1973).

Anti-nAChR mAbs were produced as described (Gomes et al., 1979) using the SP2/O-Ag 14 myeloma line, and purified from ascites fluid of hybridoma-bearing nude mice by ammonium sulfate precipitation and DEAE-cellulose chromatography.

Two different synthetic peptides corresponding to residues 127–145 (MG_1) and 187–205 (MG_2) of the α-subunit were synthesized by the solid-phase method of Merrifield (1969). The peptides were purified by HPLC and subjected to sequencing by Edman degradation.

Competition Binding Assays. ELISA Assays. Dynatech ELISA plates were coated overnight at 4 °C with solubilized nAChR (10 nM), followed by blocking with 1% BSA in PBS for 60 min at room temperature. After being washed 3 times with PBS–0.05% Tween, the plates were incubated for 2 h with the different ligands in PBS–0.02% BSA, followed by addition of the mAbs in PBS–0.02% BSA. After incubation for 2 h at room temperature, the plates were washed 3 times with PBS–0.05% Tween, and peroxidase-labeled goat anti-rat IgG (diluted 1:500 in PBS–0.02% BSA) was added for 60 min. After three washings, the substrate solution was added for 15 min (Bio-Rad peroxidase substrate kit), and the extent of enzymatic reaction was determined by reading the absorbance at 405 nm.

Rapid Centrifugation Binding Assays. ^{125}I-BGTx Binding Assays. nAChR membranes (20 nM in BGTx binding sites) in assay buffer (10 mM Hepes, pH 7.5, and 100 mM $NaNO_3$) were preincubated overnight at 4 °C with a stoichiometric amount of mAbs, followed by incubation for 2–60 min with a stoichiometric amount of ^{125}I-BGTx. The reaction was stopped by addition of 20-fold excess of cold CBTx, and the mixture was immediately centrifuged in an air-driven centrifuge at 35 psi for 2 min. Determination of free and bound ^{125}I-BGTx was performed directly in a Beckman γ-counter.

[^{14}C]BGTx Binding Assay. nAChR membranes (250 nM in BGTx binding sites) in assay buffer were preincubated overnight at 4 °C with stoichiometric amounts of mAbs, followed by the addition for 2–60 min of a stoichiometric

amount of [^{14}C]BGTx. The reaction was stopped by addition of 20-fold excess of cold BGTx, and the amount of [^{14}C]BGTx bound to the membranes was determined by the DEAE filter disk assay (Schmidt & Raftery, 1973).

Binding Assay of mAbs to α-Subunit. Pure α-subunit was obtained by preparative SDS–PAGE of alkaline-extracted nAChR membranes. After the gels were stained for 30 min with Coomassie Blue, the band corresponding to the α-subunit was excised and placed in the wells of an Isco electroelution chamber. The electroelution was performed for 12 h at 70 V in electrophoresis buffer (0.025 M Tris–0.19 M glycine, pH 8.3, and 0.2% SDS), after which the subunit was carboxymethylated (Garcia-Borron et al., 1987), dialyzed overnight against 2 L of 0.1% SDS in water, lyophilized, and stored at −20 °C until needed. To remove the detergent, the sample was dissolved in 1 mL of water and precipitated with 20 mL of cold acetone. After 1 h at 4 °C, the subunit was collected by centrifugation, resuspended in 44% formic acid to a concentration of 5–10 μg/mL, and added directly to the ELISA plates. The following procedure is a modification of the assay described by Tzartos et al. (1988). Volumes throughout the tests were 100 μL. After the plates were cooled overnight at 4 °C and washed 3 times with phosphate buffer (10 mM sodium phosphate buffer, pH 7.5), they were treated with 1% BSA in PBS. After three washes with PBS–0.05% Tween, the mAbs were incubated for 2.5 h (250 nM in Tris buffer–0.2% BSA). The plates were washed, rabbit anti-rat IgG (0.01% in Tris buffer–0.2% BSA) was added for 1 h, the plates were washed again, and each well was incubated for 30 min with ^{125}I protein A (10^5 cpm per well). After the wells were washed, the remaining radioactivity was removed with 2% SDS and counted in a Beckman γ-counter. To measure the competition between mAbs and BGTx, each well was incubated for 2 h with 3 μM BGTx prior to the addition of the mAbs.

Binding Assay of mAbs to Synthetic Peptides. The assay was performed by a solid-phase radioimmunoassay as described in the previous section, which is a modification of the procedure described by Tzartos et al. (1988); 25 μg/mL synthetic peptides in 44% formic acid were coated overnight at 4 °C. The mAb concentration in the assay was 100 nM, and the binding of the mAbs was performed in PBS–0.2% BSA. To measure competition between mAbs and BGTx, each well was incubated for 2 h with 28 μM BGTx prior to the addition of the mAbs.

Steady-State Fluorescence Titrations. All the steady-state fluorescence measurements were made on an SLM 8000C Aminco spectrofluorometer interfaced to an IBM PC/XT computer for control of the instrument and for data storage and manipulation. Fluorescence was detected by photon counting, and the emission spectra were automatically corrected by the instrument. The samples were continuously stirred, and, usually, 8-nm slits were used both for excitation and for emission.

Steady-State Fluorescence Polarization. Fluorescence polarization measurements were mady by simultaneously observing the vertical and horizontal emission from the sample using a T-optics format. Scattered light was effectively eliminated by using SLM concave grating holographic monochromators and the appropriate filters (Corning 2-63). The instrument displayed directly the data in polarization and anisotropy units.

Stopped-Flow Fluorescence Spectroscopy. Rapid kinetic measurements were carried out in a stopped-flow photometer manufactured by Kinetic Instruments (dead time 2 ms) connected to a Nicolet 3091 oscilloscope. Excitation was at 485 nm, and emission was monitored by using a Corning 3-69 filter. Further experimental details are given in the figure legends.

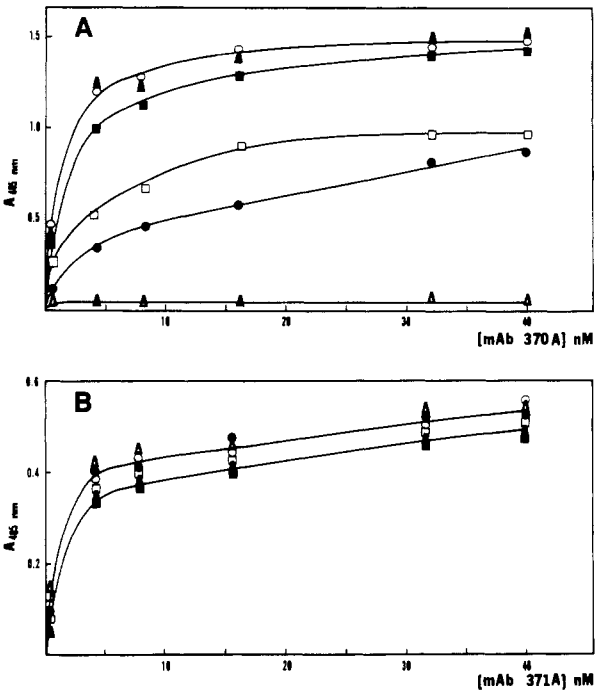

FIGURE 1: Effect of cholinergic ligands on the binding of mAb370A and mAb371A to solubilized nAChR. The ELISA competition assay was performed as described under Experimental Procedures, using as antigen solubilized nAChR (10 nM in BGTx binding sites). (A) Binding of mAb370A. (B) Binding of mAb371A. The assay was performed in the absence of ligands (○) or in the presence of 100 μM CbCh (●), 1 μM CBTx (△), 10 μM *d*-tubocurarine (▲), 100 μM decamethonium (□), or 100 μM hexamethonium (■).

Results

Competition between mAbs and nAChR Ligands. To investigate if binding of mAb370A and mAb371A to nAChR was prevented by binding of nAChR ligands, ELISA competition assays were carried out using solubilized nAChR as antigen. As shown in Figure 1, preincubation of the receptor with agonists (CbCh and decamethonium) strongly inhibited binding of mAb370A, but not of mAb371A. Conversely, CbCh binding was inhibited by preincubation of nAChR membranes with mAb370A but not by preincubation with mAb371A (Donnelly et al., 1984). In addition, preincubation of nAChR with antagonists (*d*-tubocurarine and hexamethonium, Figure 1), IMIP, or the local anesthetics tetracaine and dibucaine had no significant effect on the binding of both mAbs (results not shown). Also, as shown in Figure 1, preincubation of the receptor with CBTx completely inhibited binding of mAb370A but had no effect on binding of mAb371A. Rapid centrifugation binding assays were carried out to determine if the time course of BGTx binding (either ^{125}I or ^{14}C labeled) to nAChR membranes was affected by preincubation with the mAbs. nAChR–mAb370A complexes bound only 15% of toxin after 5 min of incubation (with respect to control nAChR membranes) and 27% after 60 min. By contrast, nAChR–mAb371A complexes bound equal amounts of toxin with respect to control nAChR membranes (results not shown).

Binding of mAbs to α-Subunit and Synthetic Peptides. Solid-phase radioimmunoassays were carried out to determine the binding specificities of the mAbs to purified α-subunit and two synthetic peptides. With pure α-subunit as antigen, three mAbs, mAb370A, mAb572, which inhibits CbCh binding (Mihovilovic & Richman, 1987), and mAb371A, were shown

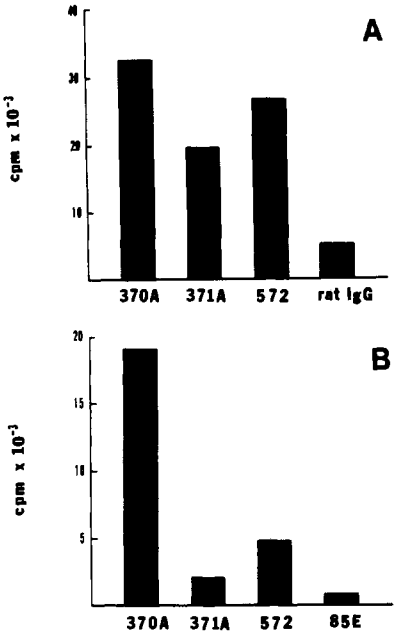

FIGURE 2: Binding of mAbs to purified α-subunit (A) and to synthetic peptide MG_2 (B). The solid-phase radioimmunoassay was performed as described under Experimental Procedures, using as antigen 0.5–1 μg of α-subunit (A) or 2.5 μg of synthetic peptide MG_2. The concentrations of mAbs were 100 nM. Binding of mAbs to synthetic peptide MG_1 was always less than 10% of the binding to MG_2.

to bind (Figure 2A). In addition, preincubation of the α-subunit with BGTx, in solution, reduced by 50% the binding of mAb370A and mAb572 but had no effect on the binding of mAb371A (results not shown).

In Figure 2B, we present the results obtained with the peptide MG_2, which corresponds to the sequence 187–205 of the α-subunit. It is known that the BGTx binding site is localized between residues 173–204 (Wilson et al., 1985), 182–198 (Mulac-Jericevic & Atassi, 1986), 185–196 (Neumann et al., 1986a), and 185–199 (Ratman et al., 1986a; Ralston et al., 1987) of the α-subunit. Only mAb370A bound significantly to the peptides; mAb572 bound with low affinity, and mAb371A did not bind. mAb 85E, a δ-subunit-specific mAb (Blair et al., 1987), was used as a control. Preincubation of MG_2 peptide with BGTx reduced by 60% the binding of mAb370A and mAb572 (results not shown). By contrast, when the synthetic peptide MG_1, which corresponds to the sequence 127–145 of the α-subunit, was the antigen, binding of any mAb was less than 10% the total for MG_2.

Since mAb370A appears to bind to a different epitope from that recognized by mAb371A, two different experimental approaches were used to attempt to identify the α-subunit region containing the latter epitope: (a) blotting of peptides obtained by digestion of purified α-subunit with V_8 protease, and subsequent immunodetection either with peroxidase-labeled second antibody or with ^{125}I protein A; (b) solid-phase radioimmunoassay of HPLC-separated peptides obtained by CNBr digestion of pure α-subunit. None of the peptides generated were recognized by mAb371A.

Effect of mAbs on the Association of Ethidium to nAChR Membranes. (I) Steady-State Fluorescence Titrations. Two features make ethidium bromide a useful probe in the study of nAChR structure–function relationships: (a) it binds with high affinity to the nAChR molecule and can be displaced by noncompetitive inhibitors such as phencyclidine and local anesthetics (Herz et al., 1987); (b) upon binding of agonists, a change in the fluorescence signal is detected (Quast et al., 1979; Schimerlik et al., 1979a,b; Herz et al., 1987). Therefore,

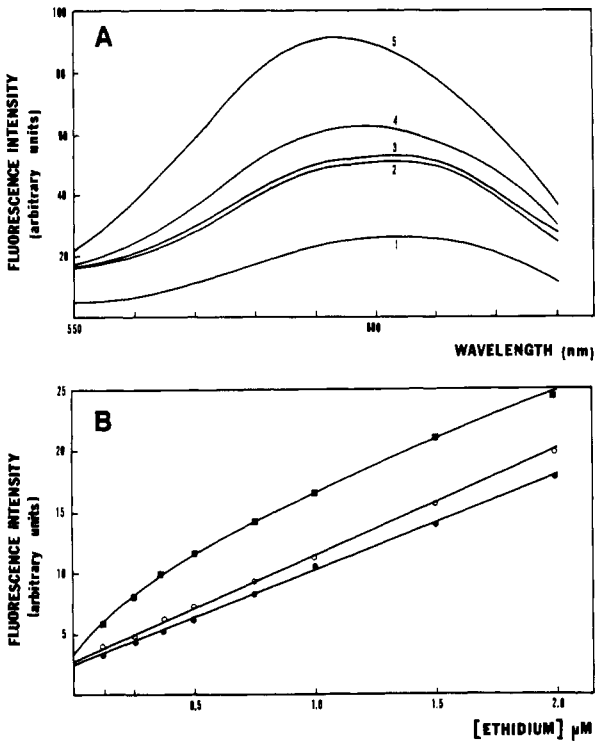

FIGURE 3: (A) Effect of CbCh, IMIP, mAb370A, and mAb371A on the fluorescence emission spectra of ethidium. The nAChR concentration was 0.2 μM in BGTx binding sites in 10 mM Hepes, pH 7.5, and 100 mM $NaNO_3$. Excitation wavelength was 485 nm. (1) 1 μM ethidium in buffer; (2) nAChR membranes preincubated for 15 min with 100 μM IMIP prior to the addition of 1 μM ethidium; (3) same as (2), with final addition of 100 μM CbCh; (4) nAChR membranes incubated with 1 μM ethidium; similar spectra were obtained by preincubation with mAb370A and mAb371A; (5) same as (4), with final addition of 100 μM CbCh. (B) Fluorescence titration of nAChR alkaline-extracted membranes with ethidium. The nAChR concentration was 0.1 μM in BGTx binding sites. All the samples were in 10 mM Hepes, pH 7.5, and 100 mM $NaNO_3$, excitation wavelength was at 485 nm, and fluorescence emission was measured at 595 nm. (●) nAChR membranes preincubated with 100 μM IMIP followed by addition of ethidium, and 100 μM CbCh; (○) nAChR membranes and ethidium. Similar titrations were obtained by preincubation with 0.2 μM mAb370A or mAb371A; (■) nAChR membranes with addition, after ethidium, of 100 μM CbCh.

we have used the fluorescence signal of this probe to study the interactions between cholinergic ligands and the nAChR molecule, and the effect of mAb370A and mAb371A on these interactions.

In Figure 3A, we present the emission fluorescence spectra of ethidium in different experimental situations: Binding of the probe to nAChR membranes increases the intensity of fluorescence, and there is a blue shift in the emission maximum from 615 to 600 nm. Addition of CbCh produces a high subsequent increase in the fluorescence intensity of the probe and a concomitant blue shift in the emission maximum from 600 to 595 nm. Preincubation of nAChR–membranes with IMIP before adding the probe results in a lower fluorescence intensity with respect to control membranes and no further increase by subsequent addition of CbCh. Similar results were obtained by preincubating the membranes with the local anesthetics tetracaine and dibucaine (results not shown). No increases in the ethidium fluorescence were detected after addition of CbCh to a preparation of solubilized receptor incubated with the probe (results not shown).

Further characterization of the interaction of ethidium with nAChR membranes was carried out by fluorescence titrations, as shown in Figure 3B. Binding of the probe to the membranes showed a saturable component superimposed with a linear

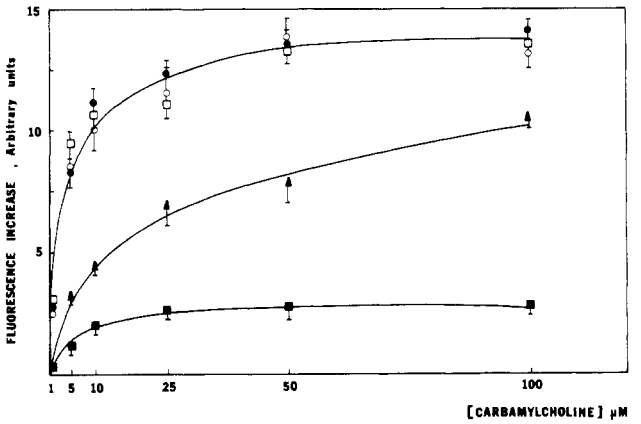

FIGURE 4: Effect of mAbs on the agonist-induced increase in the fluorescence intensity of bound ethidium. nAChR alkaline-extracted membranes (0.2 μM in BGTx binding sites) in 10 mM Hepes, pH 7.5, and 100 mM $NaNO_3$ were preincubated with 0.3 μM mAbs for 4 h followed by addition of 1 μM ethidium for 1 min. The fluorescence intensity of each sample was recorded before and after addition of CbCh (the recording was made after 1 min of incubation). Excitation wavelength was at 485 nm, and emission fluorescence was measured at 595 nm. Fluorescence intensity: fluorescence intensity after addition of CbCh minus fluorescence intensity before addition of CbCh. (○) No mAb; (●) preincubation with mAb371A; (□) preincubation with rat IgG; (▲) preincubation with mAb572; (■) preincubation with mAb370A.

nonspecific component; this nonspecific component was identified by titrations of nAChR–membranes preincubated with IMIP (or dibucaine). The subtraction of both titrations resolves the saturable binding component corresponding to the interaction of ethidium with the nAChR molecule (Herz et al., 1987). After preincubation of the nAChR membranes with mAb370A or mAb371A, we obtained titration patterns similar to those obtained with control membranes. The effect of the mAbs on the agonist-induced increase in the fluorescence intensity of ethidium is shown in Figure 4: mAb370A almost completely blocks the increase induced by CbCh, and mAb572 produces a similar effect, although to a lower extent. Both results are consistent with the previous kinetic experiments and reflect the competition between these mAbs and CbCh. By contrast, mAb371A did not prevent the increase in the fluorescence signal induced by the agonist. These results indicate that (a) mAb371A does not block binding of CbCh to the nAChR molecule, and, most significantly, (b) it does not uncouple the interactions between the agonist binding site and the ethidium binding site.

(II) Steady-State Fluorescence Polarization Measurements. The polarization value of ethidium in buffer is extremely low ($P = 0.01$), as expected for a small fluorophore in rapid isotropic motion, and this value increases after binding to the nAChR membranes (Table I). A further increase is observed after addition of CbCh, but it can be prevented by preincubation with IMIP before adding the agonist. On the other hand, the polarization value of a nAChR sample containing ethidium and CbCh was decreased after incubation with IMIP (results not shown). In Table I, we show that both mAb370A and mAb572 blocked the agonist-induced increase in the fluorescence polarization of ethidium; by contrast, mAb371A did not block that increase.

(III) Stopped-Flow Rapid Kinetic Measurements. The association of ethidium to nAChR–membranes was monitored by stopped-flow techniques, as Quast et al. (1979) used a similar experimental approach to study the kinetics of binding of agonists to nAChR membranes.

Figure 5A shows our results: In these conditions, a single-exponential phase is observed with a half-life time in the second range, which corresponds to that described by Quast et al. (1979) for high concentrations of agonists. Although the time-dependent association of ethidium to nAChR membranes is similar in presence or absence of CbCh, we observe an agonist-dependent increase in the fluorescence intensity of

Table I: Fluorescence Polarization for Ethidium Bound to nAChR Alkaline-Extracted Membranes[a]

nAChR membranes	[CbCh] (μM)		
	none	2	20
ethidium[b]	0.210	0.265	0.287
IMIP[c]	0.205		0.206
mAb371A[d]	0.217	0.264	0.284
mAb572[d]	0.213	0.236	0.254
mAb370A[d]	0.205	0.217	0.231

[a] Polarization values were determined as described under Experimental Procedures with excitation at 520 nm and emission at 600 nm. [b] nAChR membranes (0.1 μM in BGTx binding sites) incubated with 0.2 μM ethidium. [c] nAChR membranes incubated with 20 μM IMIP before addition of 0.2 μM ethidium. [d] nAChR membranes incubated with a 0.15 μM sample of the corresponding mAb before addition of 0.2 μM ethidium.

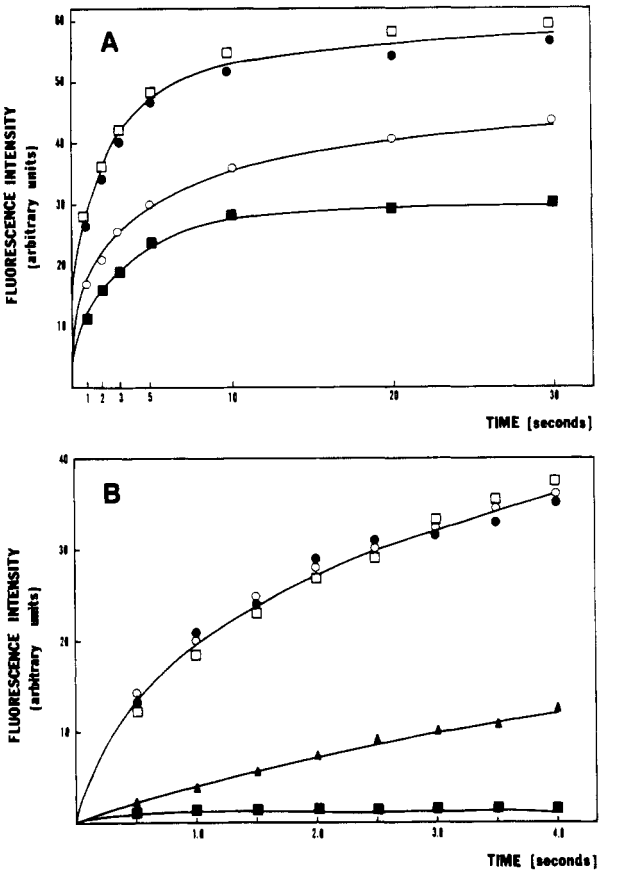

FIGURE 5: Kinetics of association of ethidium to nAChR membranes measured by stopped-flow fluorescence spectroscopy. Each point represents the average of three different determinations, and all the experiments were carried out in 10 mM Hepes, pH 7.5, and 100 mM $NaNO_3$. (A) Slow time scale: nAChR membranes (1 μM in BGTx binding sites) in the first syringe were mixed with 10 μM ethidium in the second. (○) Control membranes; (●) same as before, but with 100 μM CbCh in the second syringe; (■) membranes preincubated for 15 min with 100 μM IMIP; (□) membranes preincubated with 100 μM CbCh for 30 min (desensitized state). (B) Fast time scale: effect of mAb370A and mAb371A. nAChR membranes (1 μM in BGTx binding sites) and 5 μM ethidium in the first syringe were mixed with 5 μM ethidium and 100 μM CbCh in the second. (○) Control membranes; (■) membranes preincubated with 15 μM CBTx for 4 h; (▲) membranes preincubated with 4 μM mAb370A; (●) membranes preincubated with 4 μM mAb371A; (□) membranes preincubated with 4 μM rat IgG.

ethidium with respect to control membranes; that increase is abolished by preincubation of the membranes with IMIP. In addition, the kinetic data obtained with simultaneous addition of CbCh to nAChR membranes is identical with those with membranes preincubated with the agonist (desensitized state).

Under different experimental conditions (Figure 5B, we add ethidium in both syringes) which resolve the specific agonist-dependent increase in the fluorescence intensity of ethidium upon binding to the nAChR, preincubation of the membranes with CBTx abolishes that increase. A similar effect is obtained with mAb370A, and by contrast, preincubation with mAb371A or rat IgG has no effect.

Discussion

Both mAb370A and mAb371A have been previously shown to inhibit the agonist-induced ion flux into nAChR vesicles, as measured by a rapid stopped-flow fluorescence quenching technique (Donnelly et al., 1984). It is also known (Blair et al., 1987) that mAb371A binds to the α-subunit of the nAChR, and we have confirmed that observation by a solid-phase radioimmunoassay with α-subunit as antigen. With that technique, we also detected that mAb370A recognizes the α-subunit as well. Investigation of the ability of the mAbs for specifically recognizing two synthetic peptides, MG_1 (residues 127–145) and MG_2 (residues 187–205) of the α-subunit primary structure, shows that only the MG_2 peptide, the sequence known to contain at least part of the BGTx binding site (Mulac-Jericevic & Atassi, 1986; Ratnam et al., 1986a; Ralston et al., 1987; Neumann et al., 1986b; Giraudat et al., 1987), is also specifically recognized by mAb370A, indicating that at least part of its epitope is contained within that sequence. By contrast, neither of the peptides were recognized by mAb371A. Our attempts to characterize the mAb371A epitope by generating a set of peptides by proteolytic digestion of the α-subunit failed to detect binding of the mAb to any of these fragments. However, it should be mentioned that limited proteolytic cleavage of the purified α-subunit generates an 8-kDa peptide that can be immunoprecipitated by mAb371A (Donnelly-Roberts, 1986); unfortunately, this peptide is too long to attempt the identification of the epitope by N-terminal sequencing, and the failure of the antibody smaller fragments suggests that a critical size, or combination of sequences, is necessary for the creation of substantial structural components for the specific epitope detected by mAb371A. On the other hand, by using fluorescently labeled mAbs, we have observed simultaneous binding of mAb371A and mAb370A to the nAChR (not shown). Taking into account the relative sizes of the mAb molecules and the exposed area of the α-subunit protein, and recalling that mAbs against lysozyme epitopes occupy surface areas of about 700 Å^2 (Davies et al., 1988), our observation for simultaneous binding of the two mAbs also suggests that the binding site for mAb371A should be far apart from the toxin binding site in the nAChR surface.

There are at least three independent mechanisms and combinations thereof by which the mAbs could inhibit the agonist-induced ion flux into nAChR vesicles: (a) by direct competition with the cholinergic agonists; (b) by direct steric blockade of the ion channel; or (c) by altering conformational events in the nAChR ligand recognition and ion channel opening cycle.

The results obtained from competition experiments between the mAbs and nAChR ligands indicated that (a) cholinergic agonists (CbCh and decamethonium) and snake toxins (BGTx and CBTx) compete with mAb370A, but not with mAb371A, for binding to the receptor, and (b) competitive antagonists (hexamethonium and *d*-tubocurarine) do not inhibit binding of either mAb. Although such a result was expected for mAb371A, the inability of hexamethonium to compete with mAb370A has a less obvious interpretation. Our results lend some support to the hypothesis that the receptor sites responsible for binding of cholinergic agonists and some competitive antagonists might not be identical, but rather conformationally coupled (Fels et al., 1986). (c) The noncompetitive antagonists did not compete with either mAb for binding to the solubilized receptor.

Binding of ethidium to nAChR membranes results in a blue shift in the emission maximum of the probe, indicating that the bound ethidium resides in a hydrophobic environment with limited accessibility to water molecules (Herz et al., 1987). In addition, binding of agonist triggers a probable conformational change in the receptor that results in an increase in the fluorescence signal of the probe. It is uncertain, however, whether this change reflects an increase in the quantum yield of preexisting ethidium–nAChR complexes (Schimerlik et al., 1979a) or, as recently suggested, an increase in the fraction of ethidium specifically bound to the nAChR (Herz et al., 1987). Moreover, there is more controversy concerning the location of the ethidium binding site within the nAChR molecule. Herz et al. (1987) showed that noncompetitive inhibitor ligands, such as phencyclidine and histrionicotoxin, were able to dissociate the specifically bound ethidium from nAChR membranes, and they concluded that there is a common binding site for all these ligands located probably within the ion channel. It has also been postulated that ethidium might bind to other still unidentified sites (Schimerlik et al., 1979a). In any case, we observe that (a) the agonist-induced increase in the fluorescence signal of ethidium is specific for nAChR membranes and does not take place with solubilized receptor, and (b) for a given concentration of agonist, the kinetics of the fluorescence increase are the same whether the receptor is preincubated with CbCh and then exposed to ethidium or simultaneously mixed with CbCh and ethidium. These observations imply that any conformational transition sensed by ethidium would require the preservation of the receptor native state with an unaltered membrane–lipid interface but should not be obligatorily related to the change from the resting to desensitized state. Binding of mAb371A to nAchR membranes neither competes with ethidium nor stabilizes the receptor in a conformation with an altered affinity for the fluorescent probe. Moreover, it does not uncouple agonist binding and conformational rearrangements of the ethidium binding sites. On the other hand, mAb370A blocks the agonist-induced increase in the fluorescence signal by interfering with binding of the agonist.

The polarization value of ethidium when bound to nAChR membranes suggests that the probe is somewhat immobilized (Herz et al., 1987), and since the polarization increases in the presence of agonist, it suggests then that a higher amount of the probe may bind to the receptor. Because only binding of mAb370A inhibits this agonist-induced increase in the ethidium polarization, we feel mAb370A is a competitive inhibitor.

The kinetic traces for the ethidium fluorescence obtained by simultaneously mixing agonist with nAChR membranes were identical with that obtained for desensitized receptor. By contrast, the rate of binding of phencyclidine (followed by a manual filtration assay) to nAChR membranes is much faster in the presence of agonist than with desensitized receptor (Miguel A. Chinchetru, Jose C. Garcia-Borron, David P. Richman, and Marino Martinez-Carrion, unpublished observations). As discussed above, it has been proposed either

that both ethidium and phencyclidine bind with high affinity to the noncompetitive binding site (Herz et al., 1987) or, alternatively, that the ethidium would bind to a different binding site (Schimerlik et al., 1979a) from that responsible for phencyclidine binding. We cannot exclude any of the alternatives, although our results show that the kinetic values for association of these ligands are different. Yet, stopped-flow results show that binding of CBTx or mAb370A, but not mAb371A, inhibits the increase in the ethidium signal, which supports the proposed competitive behavior of mAb370A.

The mechanism by which mAb371A acts seems more complicated. In a previous report (Donnelly et al., 1984), it was shown that the presence of the mAb does not affect the ability of the receptor to undergo reversible sensitization–desensitization affinity transitions, as measured by a time-dependent BGTx binding assay and, as described in this work, it does not uncouple the interactions between the agonist binding site and the ethidium binding site. Thus, a mechanism based on a direct steric blockade of the ion channel by mAb371A is disfavored. At present, the most likely explanation is that mAb371A would interfere with a ligand-induced conformational change required for channel activation, but unrelated to either desensitization or to the conformational transition responsible for the increase in the fluorescence of ethidium. A different explanation is that binding of mAb371A can induce a conformational change in nAChR that shifts an open–close channel equilibrium toward the closed conformation. There are recent precedents in the interactions of antibodies with at least two proteins, neuraminidase and cytochrome *c*, for conformational changes affecting regions of the protein antigen distant from the epitope binding sites (Colman et al., 1987; Collawn et al., 1988). These new conformations can either be induced by the monoclonal antibody (Colman et al., 1987) or be detected by an altered affinity of the monoclonal antibody for the changed conformation (Collawn et al., 1988). We may not have appropriate probes to detect such changes since they do not necessarily involve the cholinergic binding sites of nAChR to which most of the above-described techniques were directed. In any case, the noncompetitive blockade of nAChR by mAb371A emphasizes the complexity of the conformational rearrangements triggered by ligand binding to the receptor and illustrates the involvement in the receptor's conformational cycle of areas in the α-subunit different from those containing the binding site for classical cholinergic ligands. Furthermore, this work stresses the concept that in the autoimmune disease *Myasthenia gravis* antibodies produced against nAChR can exert their receptor function blockade by different mechanisms. Particularly those that are specific for epitopes distant from the toxin (cholinergic) binding site(s) should not be neglected as they can significantly impair nAChR function through mechanisms distinct to competitive inhibition with the neurotransmitter.

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Thermodynamics of Phospholipid Bilayer Assembly

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ABSTRACT: Thermodynamic properties of bilayer assembly have been obtained from measurements of the solubility of the sodium salt of dimyristoylphosphatidylglycerol (DMPG) in water. The standard free energy of bilayer assembly $\Delta G°_a$ is shown to be $RT \ln Xs + zF\Psi_0$ where Xs is the mole fraction of dissolved lipid, F is the Faraday constant, z is the valence of the counterion (Na^+), and Ψ_0 is the electrical double-layer potential of the ionized bilayer. The function $d \ln Xs/dT$ was found to be discontinuous at 24 °C, the gel–liquid-crystal transition temperature (T_m) for DMPG. This function was unaffected when solubilities were measured in 0.001 M NaCl solutions; thus, Ψ_0 is constant in the experimental temperature interval (4–40 °C). Using a value of $\Psi_0 = -180$ mV [Eisenberg et al. (1979) *Biochemistry 18*, 5213–5223], and the temperature dependence of $\Delta G°_a$, values for $\Delta H°_a$ and $\Delta S°_a$ at 24 °C were calculated for the gel and liquid-crystal states of DMPG. For the gel, $\Delta H°_a$ and $T\Delta S°_a$ are −26.2 and −12.7 kcal/mol, respectively; for the liquid-crystal, $\Delta H°_a$ and $T\Delta S°_a$ are −19.2 and −5.7 kcal/mol, respectively. The calculated value for the latent heat of the gel–liquid-crystal transition is 7 kcal/mol, in agreement with calorimetric measurements.

The process of self-assembly of phospholipid bilayers from their components in aqueous solution may be represented by the equilibrium relation

$$[\text{phospholipid}]_{\text{solution}} \rightleftharpoons [\text{phospholipid}]_{\text{bilayer}} \quad (1)$$

In principle, all the thermodynamic properties of bilayer assembly may be obtained by conventional measurements of the chemical activity of the components that participate in this equilibrium. These measurements, however, have been hampered primarily because activities of phospholipids in solution are difficult to obtain due to the very low solubility of these compounds in water. It is, therefore, understandable that