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A noncholinergic site-directed monoclonal antibody can impair agonist-induced ion flux in *Torpedo californica* acetylcholine receptor

(myasthenia gravis/ion channel/receptor function)

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ABSTRACT **We have employed several monoclonal antibodies (mAbs) directed against several regions of the acetylcholine receptor (AcChoR) to assist in the determination of the antigenic structure of this multisubunit glycoprotein and to better understand molecular events involved in the impairment of neuromuscular transmission in the autoimmune disease myasthenia gravis. Among three mAbs shown to block agonist-induced ion fluxes, mAb 371A is a putative probe of an ion channel domain(s) of the AcChoR. It appears to bind to an antigenic determinant whose structure is maintained upon treatment with sodium dodecyl sulfate, the stoichiometry of binding being of one mAb per α-bungarotoxin binding site. Binding of mAb 371A to the AcChoR does not affect binding of cholinergic agonists or antagonists (carbamoylcholine and *d*-tubocurarine) or neurotoxins (α-bungarotoxin) or the ability of membrane-bound AcChoR to undergo reversible sensitization–desensitization affinity transitions. However, this mAb inhibits agonist-induced thallium (Tl^+) influx into AcChoR-rich membrane vesicles, as measured on a millisecond time scale by means of a rapid kinetics "stopped-flow/fluorescence quenching" technique. The stoichiometry of inhibition by bound mAb 371A coincides with that for maximal binding.**

The nicotinic acetylcholine receptor (AcChoR) represents the most widely studied ion channel-linked cell membrane receptor. Its abundance in electric fishes and the availability of specific probes used in affinity purification, along with the fact that it is the target of autoimmune attack in the human disease myasthenia gravis (MG), have led to its extensive investigation over the last decade. The genes coding for this pentameric glycoprotein have been cloned and the amino acid sequences of its subunit polypeptides (α, β, γ, δ) have been inferred (1–5). The snake venom toxin α-bungarotoxin (α-Bgt) binds practically irreversibly to AcChoR and blocks binding of cholinergic ligands. These α-neurotoxin probes have provided extensive information about the α-Bgt and ligand binding sites. In contrast, relatively little is known about the ion channel and the molecular protein domains involved in its transient opening upon agonist binding. Also, a number of noncompetitive channel blockers have been described that are able to modulate cholinergic ligand binding to the AcChoR molecules and to block the opening of the ion channel (6–8). However, the binding site(s) of these probes are still ill defined and could involve the cholinergic binding site itself (6, 7).

Monoclonal anti-AcChoR antibodies represent potential site-specific probes of many regions of the AcChoR molecule, including the ion channel or its interrelated regions. Since our original production of these monoclonal antibodies (mAbs) (9) a number of groups have developed them (10–15) primarily for the study of the pathogenic mechanisms involved in MG and its animal model, experimental autoimmune MG (EAMG). Much information concerning structural features of AcChoR has also been obtained by using these biological probes (11, 15–22). We present here evidence demonstrating the usefulness of anti-AcChoR mAbs as putative probes of ionic channel function as well. Our preliminary work with Fab fragments of polyclonal anti-AcChoR IgG suggested that the AcChoR sites responsible for ion translocation as determined by a "stopped-flow/fluorescence quenching" technique may be other than those involved in ligand binding sites (23). However, there still remains uncertainty as to the extent of the polyclonal Fab inhibition and the degree of saturation of possible selected antigenic sites since competition occurs between different antibodies for binding to their respective sites. These potential problems are, in principle, alleviated through the use of mAbs with which we can achieve both specificity and saturation of individual antigenic determinants.

METHODS

Materials. Frozen *Torpedo californica* electroplax was purchased from Pacific Biomarine (Venice, CA). α-Bgt was purified from *Bungarus multicinctus* (Sigma) by the method of Clark *et al.* (24). Chemicals were obtained from the following sources: carbamoylcholine (CbmCho) chloride, *d*-tubocurarine chloride, and decamethonium bromide, from Sigma; ^{125}I-labeled α-Bgt (^{125}I-α-Bgt), from New England Nuclear; 1,3,6,8-pyrenetetrasulfonic acid (PTSA), from Eastman Kodak; thallium nitrate, from Alfa–Ventron (Danvers, MA); and Cbm[*methyl*-^{3}H]Cho, from Amersham. All other reagents were analytical grade.

Production of Anti-AcChoR mAb. Anti-AcChoR mAbs were produced, as described (9), by development of hybridomas from spleen cells of Lewis rats immunized with solubilized AcChoR purified from the electric tissue of *T. californica*. These cells were fused with the nonsecreting mouse cell line SP 2/0-Ag 14. Hybridomas were screened by using a passive hemagglutination assay with solubilized *Torpedo* AcChoR as the antigen, also assessing the ability of α-Bgt to inhibit hemagglutination. mAb IgG was purified from ascites fluid of hybridoma-bearing nude mice by conventional techniques using ammonium sulfate precipitation and DEAE-cellulose chromatography (25). mAb IgG was purified to homogeneity, as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis (26) and sucrose density-gradient sedimentation.

Preparation of AcChoR-Rich Membranes. AcChoR-enriched membrane vesicles were purified from frozen *T. californica* electroplax according to published procedures (27).

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Abbreviations: MG, myasthenia gravis; AcChoR, acetylcholine receptor; α-Bgt, α-bungarotoxin; EAMG, experimental autoimmune MG; mAb, monoclonal antibody; CbmCho, carbamoylcholine; PTSA, 1,3,6,8-pyrenetetrasulfonic acid.

Alkaline extraction of this membrane preparation was performed by a modification of the method of Neubig *et al.* (28). Membranes in 10 mM Hepes, pH 7.4/100 mM $NaNO_3$ were diluted with distilled water to ≈0.5–0.6 mg of protein/ml and titrated with 1 M NaOH to a final pH value of 11.0, followed by continuous stirring for 1 hr at 4°C (23). These alkaline-extracted membranes exhibit specific activities of 30–35 μg of α-Bgt bound per mg of protein, as determined by the DEAE-cellulose filter disk assay (29). Protein concentrations were determined by the method of Lowry *et al.* (30).

Preincubation of mAb with AcChoR-Enriched Membrane Vesicles. Alkaline-extracted AcChoR-enriched membrane vesicles in 10 mM Hepes, pH 7.4/100 mM $NaNO_3$ were incubated for 1 hr at room temperature, then overnight at 4°C, with the appropriate amount of mAb per α-Bgt site to achieve the desired stoichiometric ratio. These incubation conditions were found to provide maximal antibody binding when a polyclonal antibody population was used (23, 31).

Loading of Fluorophore. AcChoR membrane vesicles with and without bound mAbs were loaded with the PTSA fluorophore by using a rapid-freezing/slow-thawing technique (27). Removal of the external fluorophore from the intravesicularly trapped fluorophore was accomplished by chromatography on a Sepharose 6MB column (30 × 1 cm). The AcChoR membranes loaded with the fluorophore were collected and submitted to the stopped-flow spectrophotometer. Presence of mAb bound to these membranes was ascertained by a positive ^{125}I-labeled protein A assay as described (23).

Stopped-Flow/Fluorescence Quenching Spectroscopy. Experiments have already been performed in our laboratory using the fast kinetics stopped-flow/fluorescence quenching technique to study the effects of polyclonal anti-AcChoR Fab fragments on the cation translocation capabilities of AcChoR-enriched membranes (23). This stopped-flow technique, which is a modification of the procedure of Moore and Raftery (32), is based on the fluorescence quenching of an intravesicularly entrapped hydrophilic fluorophore, PTSA, by an externally added cation quencher, thallium. As Tl^+ permeates into the vesicles, in response to cholinergic agonist binding, collisional quenching occurs between Tl^+ and the trapped PTSA. This Tl^+ "influx" is spectroscopically monitored as a time-dependent decrease in the fluorescence emitted by PTSA within a millisecond (ms) time scale. The spectroscopic traces are computer fitted to the Stern–Volmer equation governing the collisional quenching phenomena.

CbmCho Binding. Binding of Cbm[*methyl*-^{3}H]Cho was determined by a centrifugation assay (34) in 10 mM sodium phosphate, pH 7.5/100 mM NaCl/0.1 mM EDTA/0.01 mM phenylmethylsulfonyl fluoride/0.02% NaN_3 buffer at concentrations ranging from 1.0×10^{-7} M to 35.0×10^{-7} M.

Desensitization Measurements of mAb 371A–AcChoR Complexes. Desensitization of AcChoR membranes incubated with and without mAb 371A was followed by monitoring the time-dependent binding of ^{125}I-α-BGT as described (31), except that the buffer was 10 mM Hepes, pH 7.4/100 mM $NaNO_3$. In all experiments, the concentration of AcChoR was 5.7×10^{-8} M in α-Bgt binding sites and the concentration of α-Bgt was 10.8×10^{-8} M.

Measurement of mAb Binding to AcChoR Membranes. Titrations of membrane-bound AcChoR with mAbs were performed by a procedure similar to that described for the binding of polyclonal antibodies (31). However, instead of a fluorescent label, mAbs were radiolabeled with ^{125}I by the lactoperoxidase method employing a molar ratio of protein to Na^{125}I of 10:1. Radiolabeled mAbs were separated from reagents by passage through a Sephadex G-75 column equilibrated with 10 mM sodium phosphate buffer, pH 7.5/0.02% NaN_3 and further dialyzed against the same buffer containing 100 mM NaCl, 0.1 mM EDTA, and 0.01 mM phenylmethylsulfonyl fluoride. Specific activities of ^{125}I-labeled mAbs (^{125}I-mAbs) were in the 6×10^6 to 8.6×10^6 cpm/mmol range. After the centrifugation procedure previously described (31), both the supernatant and precipitated membranes were assayed for radioactivity in a Beckman γ counter. Parallel series in the absence of membrane were used as blanks. Competition experiments between radiolabeled and unlabeled mAbs showed that iodination does not affect their binding capacity. Anti-rat IgG does not interfere with ^{125}I-mAb binding.

RESULTS

Competition Experiments Between mAbs and Cholinergic Ligands. Three different mAbs were chosen for these experiments because of their different behavior. Although bound mAb 371A does not alter binding of α-Bgt to a mAb 371A–AcChoR complex, mAb 383C and mAb 247G have been shown previously to block nearly 100% and 50%, respectively, of ^{125}I-α-Bgt binding at short time periods of incubation (Table 1) (16). Additionally, binding of the cholinergic agonist CbmCho is drastically inhibited by the presence of mAb 383C but is not affected by mAb 247G or mAb 371A (Fig. 1). Also, binding of the cholinergic antagonist *d*-tubocurarine is unaffected by the presence of mAb 371A, whereas mAb 247G, although it does not block *d*-tubocurarine binding, does increase the dissociation constant for the *d*-tubocurarine binding site (34). In addition, the binding of these two mAbs is unaffected by the presence of a significant number of other well-known cholinergic agonists or antagonists (data not shown). The stoichiometry of mAb 371A binding to *Torpedo* AcChoR is one mAb bound per α-Bgt binding site (34). Furthermore, it appears that mAb 371A binds to an antigenic determinant on the AcChoR able to retain its antigenicity in both $NaDodSO_4$-denatured and native forms. Additionally, as determined by immunoblotting techniques subsequent to polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (unpublished data), mAb 371A binds solely to the α ($M_r \approx 40{,}000$) subunit of the AcChoR.

Effect of Different mAbs on the AcChoR Cation Flux Mediated by Cholinergic Agonist. Studies using the stopped-flow/fluorescence quenching procedure on AcChoR membranes preincubated with a stoichiometric ratio of 1:1 of mAb 371A, 247G, or 383C per α-Bgt binding site indicate strong inhibition of the agonist-induced Tl^+ influx by each of the three

Table 1. α-Bgt binding and agonist-induced Tl^+ fluxes in AcChoR-rich vesicles

	% α-Bgt binding*	% Tl^+ fluxes†
AcChoR (control membranes)	100.0	100.0
Complex		
AcChoR–mAb 371A	99.2 ± 3.2	<5
AcChoR–mAb 247G	55.2 ± 1.4	<5
AcChoR–mAb 383C	18.6 ± 3.8	<5
AcChoR–normal rat IgG	100.0	100.0
AcChoR–mAb D547	100.0	100.0

*AcChoR-rich vesicles (0.87×10^{-8} M in α-Bgt binding sites) were incubated overnight at 4°C with at least 5 mol excess of mAb in 10 mM sodium phosphate buffer, pH 7.4/100 mM NaCl/0.01 mM phenylmethylsulfonyl fluoride/0.02% NaN_3. Then a 2.5 molar excess of ^{125}I-α-Bgt was added and toxin binding was determined after 5 min of toxin addition. Data represent the average of two independent determinations.

†AcChoR-rich vesicles were preincubated overnight at 4°C in the presence or absence of a 1:1 molar ratio of antibody to α-Bgt binding sites in 10 mM Hepes, pH 7.4/100 mM $NaNO_3$. The following day, the vesicles were loaded with PTSA and the samples were submitted to the kinetic analysis by the stopped-flow procedure.

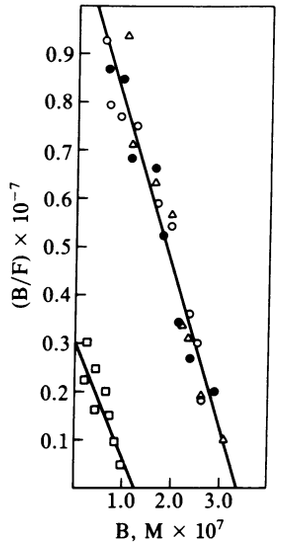

FIG. 1. Scatchard plot of CbmCho binding to membrane-bound AcChoR and preformed AcChoR–mAb complexes. Cbm[*methyl*-^{3}H]Cho binding was determined at a final AcChoR concentration of 4.1×10^{-7} M in α-Bgt binding sites. AcChoR–mAb complexes were produced employing—at least—a 1.5 molar excess of mAb over α-Bgt binding sites. Values for the dissociation constants (K_d) and stoichiometry of binding ($\bar{\mu}$) were obtained by linear regression analysis of the data. For AcChoR (○): $K_d = 3.27 \times 10^{-7}$ M, $\bar{\mu} = 0.84$ mol of CbmCho per mol of α-Bgt sites; for AcChoR–mAb 247G (●): $K_d = 3.17 \times 10^{-7}$ M, $\bar{\mu} = 0.83$ mol of CbmCho per mol of α-Bgt sites; for AcChoR–mAb 371A (△): $K_d = 2.74 \times 10^{-7}$ M, $\bar{\mu} = 0.80$ mol of CbmCho per mol of α-Bgt sites; for AcChoR–mAb 383C (□): $K_d = 4.16 \times 10^{-7}$ M, $\bar{\mu} = 0.31$ mol of CbmCho per mol of α-Bgt sites. Drawn curve for AcChoR, AcChoR–mAb 247G, and AcChoR–mAb 371A represents the average fitted curve. B, bound CbmCho; F, free CbmCho.

mAbs (Table 1). The inhibition of the agonist-induced Tl^+ influx by mAb 371A was investigated further and shown to depend upon the molar ratio of mAb bound per α-Bgt site (Fig. 2). At lower ratios, there is little inhibition of cation influx, with progressively greater inhibition at higher molar ratios until complete inhibition is achieved at approximately

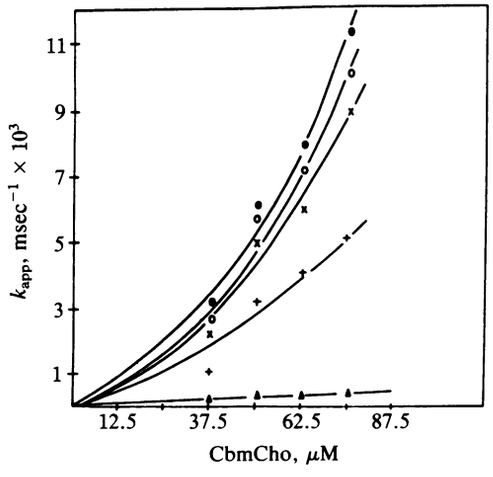

FIG. 2. Effect of bound mAb 371A on the agonist-mediated Tl^+ influx of AcChoR membrane vesicles. Apparent rate constants for cation influx were obtained by computer fitting the kinetic traces from the stopped-flow spectrometer to the Stern–Volmer equation (23). ●, Control AcChoR-rich membranes alone; ○, 0.1:1 molar ratio of mAb 371A per α-Bgt site; ×, 0.25:1 molar ratio of mAb 371A per α-Bgt site; +, 0.5:1 molar ratio of mAb 371A per α-Bgt site; ▲, 1:1 molar ratio of mAb 371A per α-Bgt site.

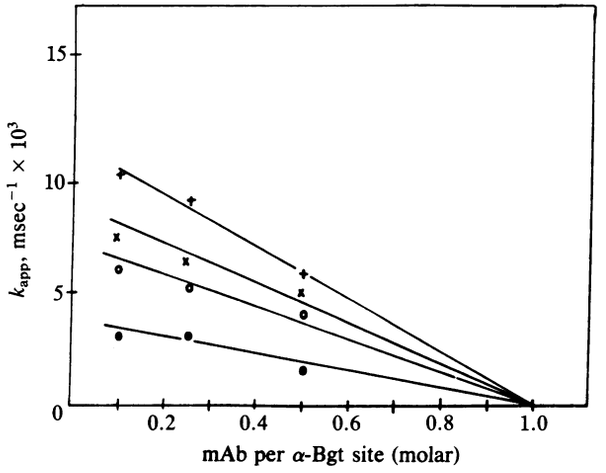

FIG. 3. Effect of varying amounts of mAb 371A on CbmCho agonist-mediated Tl^+ influx into AcChoR membrane vesicles. CbmCho concentrations were 75 μM (+), 62.5 μM (×), 50 μM (○), and 37.5 μM (●). The extrapolation to the *x*-intercept at each CbmCho concentration indicates the approximate molar ratio value of mAb 371A per α-Bgt binding site for complete inhibition.

one mAb 371A per α-Bgt binding site. Fig. 3 also shows that the same (1:1) stoichiometry of cation influx inhibition is obtained by extrapolation when plotting the observed rate constants obtained at different CbmCho concentrations against various mAb 371A/α-Bgt molar ratios. Control experiments to consider the possibility of extraneous or nonspecific immunoglobulin or other serum contaminants on membrane permeability were carried out by using polyclonal anti-rat IgG or an inert mAb line, mAb D547. As indicated in Table 1, cation permeability is unaffected by the presence of these antibodies, which were used at the same concentrations as those tested for inhibitory function.

Effects of mAb 371A on the "Desensitization" Phenomenon. Fig. 4 shows the patterns obtained from mAb 371A–AcChoR membrane complexes, indicating the characteristic reduction [as it can be obtained in the absence of antibody (31)] in the time-dependent α-Bgt binding after preincubation of the membranes with the cholinergic agonist CbmCho (31). Hence, binding of stoichiometric amounts of mAb 371A, rel-

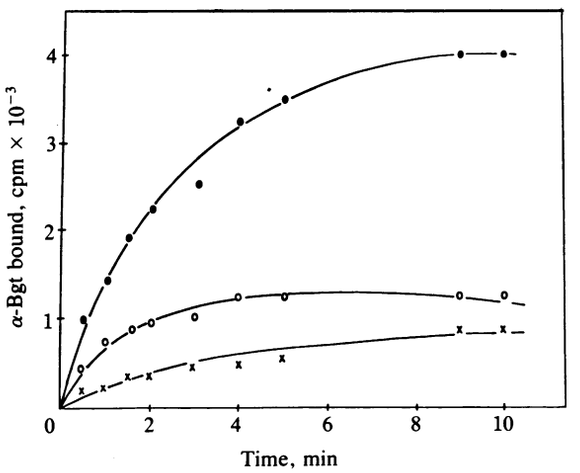

FIG. 4. Effect of mAb 371A on the time dependence of α-Bgt binding to membrane-bound AcChoR (desensitization). Membrane-enriched AcChoR vesicles were incubated with a 1:1 molar ratio of mAb 371A per α-Bgt binding site. ●, ^{125}I-α-Bgt added to the membranes at zero time; ○, 1×10^{-6} M CbmCho and ^{125}I-α-Bgt added simultaneously at zero time; ×, membranes incubated with 1×10^{-6} M CbmCho for 30 min prior to the addition of α-Bgt.

ative to α-Bgt binding sites for the AcChoR, does not diminish the ability of membrane-bound AcChoR to undergo the α-Bgt time-dependent binding process that is characteristic of agonist-induced affinity state transition akin to desensitization.

DISCUSSION

We have shown previously that in EAMG the polyclonal Fab population contains antibodies that decrease the magnitude of the desensitization-like process of time-dependent α-Bgt binding in AcChoR membranes and shift the agonist-induced (CbmCho) dose–response curve for trapped $^{22}Na^+$ efflux to lower concentrations (31). These polyclonal antibodies have also been shown to inhibit the rate of agonist-mediated Tl^+ influx mostly in a noncompetitive manner (23), thus indicating the presence of antibodies specific for "ion channel"-associated epitopes different from those that can constitute the cholinergic ligand binding site.

Our search for mAbs directed against unique determinants of the AcChoR that are involved in the activation of the ion channel started with three mAbs that exhibited unique patterns of interference with some functional properties of the AcChoR. These three mAbs fell into different categories when measuring their effects on α-Bgt binding: mAb 383C inhibited 100% of the binding, mAb 247G inhibited 50% α-Bgt binding, whereas mAb 371A did not affect α-BGT binding to the AcChoR (16). Preliminary studies using a fast kinetics assay (stopped-flow) measuring agonist-mediated ion influx revealed marked inhibition of Tl^+ influx when incubating AcChoR membrane vesicles with each of these three mAbs at a stoichiometric ratio of one mAb bound per α-Bgt site. For mAb 383C, the inhibition could be explained by its ability to block the agonist binding site, whereas inhibition of Tl^+ influx by mAb 371A was unexpected. mAb 371A inhibits Tl^+ influx in an antibody concentration-dependent fashion with inhibition gradually increasing until complete inhibition is achieved at a ratio of one mAb bound per α-Bgt binding site. The possibility that mAb 371A-mediated Tl^+ influx inhibition results from the induction of an equilibrium shift toward the high-affinity state for agonist, such as in AcChoR desensitization, was tested by measuring the effect of mAb 371A on desensitization. This mAb did not affect the ability of AcChoR membrane to undergo the affinity state transitions that lead to desensitization, at least when this was tested as a function of α-Bgt rate of binding. Therefore, mAb 371A, which binds to an AcChoR site unrelated to both ligand and toxin binding, appears to be directed to receptor domain(s) related to ion channel activity.

Initial attempts to demonstrate the ability of anti-AcChoR mAbs to block agonist-induced ion fluxes involved monitoring the extent of $^{22}Na^+$ uptake from vesicles reconstituted with AcChoR (19). Blockade was detected when preformed mAb–AcChoR complexes were employed to reconstitute the membrane system but not when mAb was added after reconstitution (19). Those results were interpreted as demonstrating a noncompetitive effect between the binding of CbmCho and the mAb (19). Yet, a reduction in the number of functional receptor molecules inserted in the reconstituted system, as well as lowered reconstitution efficiency by the pretreatment with the mAb, could have been responsible for producing the reductions in the amount of maximal $^{22}Na^+$ effluxed. On the other hand, the findings presented here demonstrate that, indeed, mAbs can directly affect functions of *native* AcChoR membrane vesicles and are consistent with our previous studies using polyclonal Fab fragments (23). These data suggest the existence in EAMG of antibodies capable of blocking intact AcChoR, not by effects on neurotransmitter recognition but through the impairment of AcChoR ion flux function—i.e., ion channel and the associated domains involved in its activation. It is of interest that concurrent with submission of this manuscript, a brief report appeared (33) on the inhibition of single channel currents of AcChoR reconstituted into lipid bilayers by a mAb reactive against the β and α subunit. The possible interference between this mAb and cholinergic ligand binding was not reported.

The manner by which mAb 371A blocks agonist-induced ion fluxes remains undetermined. It is possible that these effects occur as the result of mAb-induced conformational events in the AcChoR protein or through direct steric effects on the ion channel itself. In addition, the specificity of mAb 371A for an epitope on the α subunit of the AcChoR indicates that it is a probe of a single specific cholinergic receptor domain, a characteristic not shared by noncompetitive ligands that modulate agonist-induced ionic translocation. This property may help in the further characterization of the structure and function of these protein domains. It is particularly intriguing, if this is not a direct ion channel steric blocker, that certain surface sites in the α-chain can be "frozen" by the presence of this tightly bound mAb 371A. This would prevent the conformational change, or rotation of the subunits (20), necessary for opening the cation channel after neurotransmitter, or agonists, binding to the receptor sites.

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