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BINDING OF ANTI-ACETYLCHOLINE RECEPTOR ANTIBODIES INHIBITS THE ACETYLCHOLINE RECEPTOR MEDIATED CATION FLUX

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A fast kinetics, spectroscopic technique has been applied to the study of the transient cation flux associated to the binding of cholinergic agonist to native acetylcholine receptor (AcChR)-rich membrane vesicles in presence of anti-AcChR antibodies. The technique is based on the collisional quenching of an intravesicularly trapped fluorophore by externally added Tl^+ which substitutes for physiologically occurring cations. Presence of polyclonal Fab fragments from goat anti-AcChR antibodies bound to the membrane AcChR promotes a 80-90% inhibition on the observed rate constants of Tl^+ influx. The observed inhibition process appears to follow a non-competitive pattern between antibody and cholinergic ligand binding, suggesting that in the AcChR protein the antigenic sites responsible for ion translocation may be other than those involved in ligand binding.

In patients suffering from Myasthenia gravis (MG)[1], acetylcholine receptor (AcChR) antibodies have been detected. These antibodies have been shown to bind to: i) AcChR at the motor end plate (1,2), ii) cultured mammalian muscle cells (3), and iii) Torpedo AcChR in both solubilized and membrane-bound preparations (4-7). Presence of anti-AcChR antibodies correlates with a variety of other effects such as increased AcChR turnover, decreased AcChR electrophysiological response and an overall morphological simplification of the motor end plate (8). Normal animals injected with solubilized AcChR or AcChR-rich membrane preparations also develop the general symptoms of MG and this condition is known as experimental autoimmune MG (EAMG) (9-11). These symptoms are also shown in animals which have been injected with a variety of anti-AcChR monoclonal antibodies (12,13).

Biopsies from MG patients and EAMG animals exhibit fewer receptor-specific α-neurotoxin binding sites at the neuromuscular junction (14,15). Nevertheless,

[1] Abbreviations used are: AcChR, acetylcholine receptor; MG, Myasthenia gravis; EAMG, experimental autoimmune Myasthenia gravis; α-Bgt, α-Bungarotoxin; PTSA, 1,3,6,8-pyrene tetrasulfonate.

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most binding of neurotoxins as well as of cholinergic ligands still occurs (9,16,17) leading to the perception that MG or EAMG anti-AcChR antibodies could primarily be directed against AcChR sites other than those involved in neurotoxin and neurotransmitter binding. An exciting possibility is that some anti-AcChR antibodies could be directed against those sites in the AcChR molecule constituting the so called ion "channel" or controlling the protein conformational change leading to its formation, without impairing ligand binding. Testing of this hypothesis has traditionally involved slow determinations of only the end points of the AcChR-mediated cation translocation based on ultrafiltration or ion exchange chromatography techniques using radiolabeled cation tracers such as $^{22}Na^+$, $^{86}Rb^+$, etc. (18-21). This has lead to inconclusive and even contradictory interpretations on the potential role of antibodies.

Recently (22-24), fast kinetic techniques have been developed which allow for accurate determinations of the cation translocation events associated with activation of AcChR on the physiological time scale (milliseconds). In this communication, we have applied a modification of one of those techniques (24) to study the effect of EAMG anti-AcChR polyclonal Fab's binding on the rate of membrane ion permeation mediated by presence of neurotransmitter agonist in AcChR rich membranes.

MATERIALS AND METHODS

AcChR-enriched membrane vesicles were purified from Torpedo californica electroplax and subjected to alkaline extraction of peripheral membrane proteins (25,26). These membranes were suspended in Hepes buffer (10 mM Hepes, pH 7.4, 100 mM $NaNO_3$) and exhibited specific activity values of 30-35 µg of α-bungarotoxin (α-Bgt) bound per mg of protein.

Fab fragments from polyclonal IgG from control or AcChR-immunized goats suffering EAMG were prepared as previously described (16,17). Two different preparations (from different goats) of anti-AcChR Fab fragments were used. Both preparations exhibited similar AcChR binding activity as estimated by ELISA procedures (data not shown).

AcChR membranes were incubated 1 h at 22°C and overnight at 4°C with a 30 fold molar excess of Fab (control or anti-AcChR) over the α-Bgt binding sites. Further increase of the Fab to α-Bgt binding sites molar ratio does not result in any additional Fab binding to the membrane preparations (17). Unbound and weakly bound Fab were removed by centrifugation of the membranes prior to resuspension in Hepes buffer for submission to the fluorophore loading procedure.

Loading of the fluorophore 1,3,6,8-pyrene tetrasulfonate (PTSA) (Eastman-Kodak) (final concentration 4.3 mM) into AcChR membranes was accomplished through a rapid freezing/slow thawing technique (27). Removal of the external fluorophore from the vesicles was accomplished by chromatography on a Sepharose 6 MB (Macrobeads) column (30 x 1 cm). Collected AcChR membranes loaded with

the fluorophore were submitted to the stopped flow spectrophotometer without further dilution. The above procedures did not alter the amount of bound Fab to the AcChR membrane preparation as measured by an immunoassay procedure using rabbit anti-goat IgG (Cappel) followed by incubation with [^{125}I] protein A (New England Nuclear) of the membranes before and after the freezing/thawing and the filtration chromatography steps.

Fast kinetic measurements of Tl^+ influx through the membranes were obtained using a Durrum D-110 stopped-flow spectrophotometer set up in the fluorescence mode using the following modifications to the procedure described by Moore and Raftery (24). Excitation wavelength was 378 nm and emission of fluorescence was monitored using a Corning 3.75 filter. The stopped-flow spectrophotometer was upgraded with the addition of a PRA optical feed-back system for a better stabilization of the intensity of the exciting light. The flux process was initiated by rapid mixing (dead time was approximately 3-4 milliseconds) of the fluorophore loaded vesicles with an equal volume of 10 mM HEPES, pH 7.4, 70 mM $NaNO_3$, 30 mM $TlNO_3$ buffer containing various concentrations of cholinergic agonist (carbamylcholine). Final Tl^+ concentration upon mixing (15 mM) produces a 50% quenching of PTSA fluorescence at equilibrium conditions. The pH of all HEPES buffers was adjusted with HNO_3.

Agonist mediated-AcChR activation was monitored as a rapid influx of Tl^+ during the initial 100 milliseconds following rapid mixing in the stopped-flow spectrophotometer, therefore avoiding the concommitant occurrence of AcChR desensitization (28). Much slower passive Tl^+ "leakage" (non-agonist mediated influx) into the AcChR vesicles was spectroscopically monitored on a 25 second time scale. The spectroscopic data was stored in a Physical Data model 514A transient recorder and visualized in a Tektronic oscilloscope. The spectroscopic traces were computer fitted by an interfaced Apple microcomputer to a time dependent Stern-Volmer equation describing collisonal quenching (24).

RESULTS AND DISCUSSION

The fast kinetics, stopped-flow method used here is similar to the one described by Moore and Raftery (24) with two differences: a) the nature of the fluorophore and b) the procedure to separate fluorophore loaded vesicles from free fluorophore. As 8-amino-1,3,6-naphtalenetrisulfonate, PTSA has excellent solubility in water and this along with the four negatively charged sulfonate groups prevent its permeation through the vesicle membrane as well as its insertion into the vesicle bilayer. In fact, no significant leakage of PTSA out of the loaded vesicles occurs for at least a period of several days at 4°C. Also, the fluorescence of PTSA can be effectively quenched by added Tl^+ through a collisional dynamic quenching process, characterized by a Stern-Volmer constant of 39.4 M^{-1} at the operating temperature of 23°C. PTSA exhibits considerably higher quantum yields than the aminonaphtalenetrisulfonate probe and does not undergo significant photolysis during the experimental time of exposure to the exciting light which leaves the quenching by the added Tl^+ as accountable for the observed fluorescence decays.

We have estimated on the basis of α-Bgt sites recovery that when fluorophore loaded vesicles are separated from free, excess fluorophore, using Sephadex G-25 or G-50 (coarse grade) or Sepharose 2B or 4B, only 25-30% of the membrane vesicles are able to elute through the resin bed. Conversely, when Sepharose 6MB (macrobeads) is used, a quantitative recovery of membrane vesicles is obtained upon passing of the sample through the column.

The approach we have used to study how the presence of bound anti-AcChR antibodies affects functional properties of native AcChR has several advantages. First, polyclonal antibodies are expected to mimic in vivo effects of antibody produced during either MG or EAMG. Second, since univalent Fab fragments, which inhibit the binding of their parental IgG (16,17), are used instead of divalent IgG molecules, potential problems such as insolubility resulting from immunoprecipitation or receptor capping through crosslinking by the divalent antibody are eliminated. Also, Fab fragments are approximately one third the size of intact IgG, therefore steric factors are reduced.

Binding of polyclonal anti-AcChR Fab to AcChR membranes has two apparent effects on the permeability properties of the membranes. First, passive "leakage" of Tl^+ exhibits rate constant values of 0.12 and 0.19 S^{-1} for the control (presumed normal Fab) and anti-AcChR Fab preincubated membranes, respectively. Control membranes with no Fab behave identically to samples preincubated with normal goat Fab. In either case, this passive influx of Tl^+ represent a small contribution compared to the agonist-mediated process which is faster by about 3 orders of magnitude in its rate constant. Second, presence of bound anti-AcChR Fab fragments to AcChR membranes inhibits the agonists mediated cation flux (Figure 1). In different experiments, the inhibition of Tl^+ influx was estimated to be 80 to 90% at different carbamylcholine concentrations. However, some uncertainty remains as to the true extent of the inhibition process precisely because polyclonal Fab are used and the degree of saturation of selected sites involved in the ion translocation event in the AcChR molecule can not be ascertained under these conditions where competition among the polyclonals for antigenic sites on the AcChR has been shown to occur

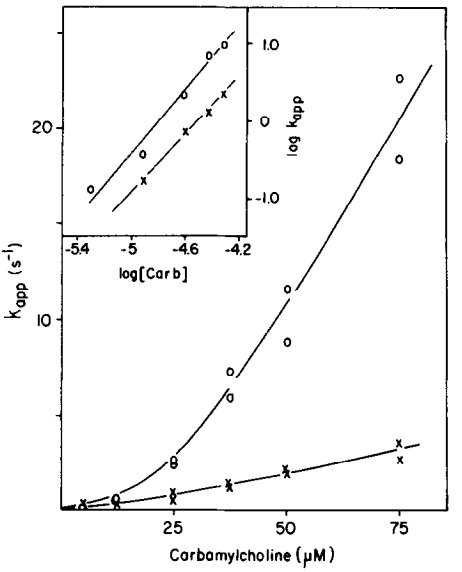

Figure 1. Dependence of the apparent Tl^+ flux rate on the concentration of carbamylcholine. Apparent rate constants were obtained from computer fitting of the kinetic traces from the stopped-flow experiments, to the time dependent Stern-Volmer equation (see ref. 24). AcChR-enriched membranes in 10 mM Hepes buffer, pH 7.4, 100 mM $NaNO_3$, were preincubated with normal goat Fab (O, control) or with anti-AcChR polyclonal Fab's (X). Insert: Double log plot of the dependence of the apparent rate constants with the concentration of agonist. Estimations for the Hill coefficients are given by the slopes (see text and Table I).

(17). Since EAMG is a polyclonal event, we have first studied the complete system which in the near future will be analyzed for the effects of individual or mixtures of monoclonal antibodies to achieve both selective binding and true saturation conditions for specific antigenic sites.

Under our experimental conditions for the concentration of Tl^+ used (final concentration, 15 mM), the highest concentration of cholinergic agonist (carbamylcholine) tested is 100 μM. The reason for this limitation is that when higher agonist concentrations are used most of the Tl^+ influx occurs within the instrument's "dead time" (3-4 msec), which prevents the proper recording of the spectroscopic trace as a well defined, time dependent fluorescence decay. To handle this problem, other authors chose to treat the membrane preparations with non-saturating concentrations of histrionicotoxin (24) or α-Bgt (25) to partly block the available ion channels so that maximal rates can be extrapolated from the observed behavior of a small population of unblocked AcChR molecules. Such procedures are unnecessary for testing the inhibitory effect

Table I

Estimated values for kinetic parameters of agonist-mediated Tl^+ influx in AcChR-membranes in the presence of bound normal goat Fab (control) and anti-AcChR Fab [a]

	control membranes	anti-AcChR Fab membranes
Hill coefficient (n)[b]	2.0	1.7
k_{app}^{max} [c]	87.6 s^{-1}	15.5 s^{-1}
K_D (carbamylcholine)[c]	1.3 x 10^{-4} M	1.11 x 10^{-4} M

[a] Results represent the average of calculated values obtained from two separate experimental determinations of Tl^+ flux rates for carbamylcholine concentrations up to 100 μM (see text).

[b] Hill coefficients (n) when $k_{app} << k_{app}^{max}$ are first calculated from the slope of a double log plot of the dependence of k_{app} vs agonist concentration.

[c] To estimate the apparent maximum rate and the dissociation constant, we rounded the Hill coefficient values to 2 in the equation $k_{app} = k_{app}^{max} [A/(A + K_D)]^n$ and analyzed the data in a plot of $k_{app}^{-1/2}$ vs 1/A, where A = agonist concentration (see ref. 30).

of bound Fab, which can be demonstrated at the lower carbamylcholine concentrations.

Procedures are also available which allow for the determination of the Hill coeficient, the maximum rate and the apparent dissociation constants from only rate constant values obtained at low agonist concentrations where $k_{app} << k_{app}^{max}$ (Table I). This approach has been used by others (29,30) and is based on the Hill equation (31). Estimated average values for k_{app}^{max} confirms the experimentally observed percentage of inhibition by anti-AcChR Fab at low agonist concentrations. Since the apparent K_D value for carbamylcholine is maintained in presence of bound Fab fragments a non-competitive behavior between anti AcChR-Fab and cholinergic agonists is suggested. Also, the value of the kinetic parameters from the "control" samples from Table I essentially agree with data obtained by different groups using a different approach: quench-flow kinetics (29,30,32). Moreover, considering existing differences in membrane purification procedures, density of AcChR within the membrane population, vesicle internal volume, etc. (32) the similarities observed are even more striking.

In conclusion, our data strongly suggests that presence of bound anti-AcChR antibody leads to blockage of the ion translocation capabilities of the AcChR. This may be one of the primary events involved in MG AcChR deterioration and is consistent with physiological recordings of diminished miniature end-plate potentials (33). Also, the apparent non-competitive behavior between Fab and cholinergic ligands and the maintenance of the affinity (K_D's) of the AcChR for cholinergic ligands as in the absence of bound antibody further suggests that the antigenic sites responsible for blockage of ion translocation on the receptor surface are different from the cholinergic binding sites. Such a proposal was the explanation for the effect of a monoclonal antibody on a reconstituted membrane system in which the IgG was preincubated with the receptor prior to reconstitution (21). In the present study, it is apparent that such antigenic site(s) must be available in native membrane preparations. Since these are membrane vesicles which exist with over 95% of "right-side" out-oriented α-Bgt sites, our results also indicate that in EAMG, antibodies are produced which interact with protein sites located in the exocytoplasmic residues of the AcChR.

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