# Effects of Monoclonal Antibodies on the Function of Acetylcholine Receptors Purified from *Torpedo californica* and Reconstituted into Vesicles†

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ABSTRACT: We tested the effects of 62 monoclonal antibodies (mAbs) to acetylcholine receptors from *Torpedo californica* on the function of receptor reconstituted into lipid vesicles. Two of these mAbs, mAbs 148 and 168, inhibited carbamylcholine-induced $^{22}Na^+$ uptake into vesicles. The rate of $^{125}I$-labeled $\alpha$-bungarotoxin ($^{125}I$-$\alpha$BGT) binding to the reconstituted liposomes was also reduced, although $^{125}I$-$\alpha$BGT binding at equilibrium was not affected. Agonist-induced desensitization of the receptor was also affected by these mAbs. mAb 148 binds to the $\beta$ subunit of receptor, and mAb 168 binds to the $\gamma$ subunit. Both mAbs bind to the cytoplasmic surface of the receptor; correspondingly, both block function when added before reconstitution, and both were found to have no effect on function when added to preformed vesicles. Their effects were not due to interference with the reconstitution process. Both mAbs were capable of cross-linking receptors. In contrast to the bivalent mAbs, monovalent Fab fragments of these two mAbs had little effect on receptor function, which suggests that the effects of the bivalent mAbs resulted primarily from cross-linking receptors.

Acetylcholine receptors from the electric organs of *Torpedo californica* are composed of four kinds of glycoprotein subunits with the subunit stoichiometry $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980). Reconstitution of purified receptor into lipid vesicles indicates that the $\alpha_2\beta\gamma\delta$ complex constitutes the basic functional unit (Huganir et al., 1979; Lindstrom et al., 1980; Anholt et al., 1980; Popot et al., 1981; Criado et al., 1982; Ochoa et al., 1983). The $\alpha$ subunits contain the physiologically significant acetylcholine binding sites, whereas the specific functional roles of the other subunits are largely unknown. All four kinds of subunits are thought to have evolved from a primordial subunit and to share a fundamentally homologous structure (Raftery et al., 1980; Conti-Tronconi et al., 1982; Noda et al., 1983). Further, it is thought that all of the subunits are oriented like barrel staves around a cation channel that runs through the center of the molecule and that part of each subunit contributes to the lining of this channel (Finer-Moore & Stroud, 1984; Noda et al., 1983; Devillers-Thiery et al., 1983; Oswald et al., 1983). Studies of the expression of subunit cDNA clones have shown that, despite their structural homologies, all four kinds of subunits are required to efficiently reconstitute receptor function (Mishina et al., 1984).

Monoclonal antibodies (mAbs)[1] provide specific probes for the structure and function of the receptor which can be prepared against many parts of the receptor molecule (Lindstrom, 1984). A number of groups have prepared mAbs directed against the acetylcholine binding sites on the extracellular surface of the receptor (Gomez et al., 1979; James et al., 1980; Mochly-Rosen & Fuchs, 1981; Watters & Maelicke, 1983). mAbs to acetylcholine binding sites obviously inhibit receptor function. The main immunogenic regions of the receptor, like its acetylcholine binding sites, are on the extracellular surface of the receptor, but mAbs bound to the main immunogenic region do not inhibit receptor function (Lindstrom et al., 1981b, 1984). mAbs made against denatured receptor subunits are most often directed at the cytoplasmic surface of the receptor (Froehner et al., 1983; Sargent et al., 1984). We have generated a library of nearly 200 mAbs against different parts of the receptors from various species (Tzartos & Lindstrom, 1980; Tzartos et al., 1981, 1983; Gullick & Lindstrom, 1983; S. Tzartos et al., unpublished results; S. Hochschwender et al., unpublished results). Here we report screening 62 of these, most of which were directed at the cytoplasmic surface, for their ability to inhibit carbamylcholine-induced influx of $^{22}Na^+$ into lipid vesicles into which receptors purified from *Torpedo californica* were reconstituted. Two mAbs were found to affect both $^{22}Na^+$ uptake and $^{125}I$-labeled $\alpha$-bungarotoxin ($^{125}I$-$\alpha$BGT) binding. These two mAbs are especially interesting because they bind to the cytoplasmic surfaces of $\beta$ and $\gamma$ subunits.

## Materials and Methods

*Monoclonal Antibodies.* mAbs were prepared by fusion of spleen cells from immunized rats with mouse or rat myeloma cell lines as previously described (Tzartos & Lindstrom, 1980; Tzartos et al., 1981; Lindstrom et al., 1981). Clones were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% serum (either horse or fetal calf), in serum-free DMEM, or in Iscove's-modified DMEM (Irvine Scientific) plus 1% fetal calf serum, 0.001% transferrin, 0.001% insulin, 90 $\mu$M 2-mercaptoethanol, and 2 mM glutamine. Receptor

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[1] Abbreviations: $^{125}I$-$\alpha$BGT, $^{125}I$-labeled $\alpha$-bungarotoxin; mAbs, monoclonal antibodies; GART, goat anti-rat IgG; IgG, immunoglobulin G; DMEM, Dulbecco's modified Eagle's medium; PBS-$N_3$, 10 mM sodium phosphate, 100 mM NaCl, and 10 mM sodium azide, pH 7.5; DEAE, diethylaminoethyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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subunit specificity was determined by using $^{125}$I-labeled subunits as previously described (Tzartos & Lindstrom, 1980; Tzartos et al., 1981; Gullick & Lindstrom, 1983) and also by ELISA assay using purified subunits bound to plastic wells and glucose oxidase conjugated affinity-purified goat anti-rat IgG as previously described (Hochschwender et al., 1984). Determination of immunoglobulin class and subclass was done by the Ouchterlony and radioimmunoassay methods using anti-rat immunoglobulin subclass antisera (Miles) and known controls.

*Purification of mAbs.* Culture supernatants were concentrated by ultrafiltration (Amicon), and the crude IgG fractions were obtained by ammonium sulfate precipitation at 45% saturation. After dissolution, dialysis, and concentration, the mAbs were affinity purified by adsorption onto goat anti-rat IgG (GART)–Sepharose CL-4B. The mAbs were then eluted either by 3 M NaSCN in PBS–$N_3$ (10 mM sodium phosphate, 100 mM NaCl, and 10 mM sodium azide, pH 7.5) or by 0.1 M triethylamine, pH 11.5. We found that alkaline elution sometimes caused less denaturation than did acid elution or NaSCN. For bulk preparation of pure mAbs, such as mAbs 148 and 168, DEAE Affi-Gel Blue (Bio-Rad) was used. The mAb was eluted by a 0–100 mM NaCl linear gradient in 20 mM Tris-HCl, pH 8.0, buffer. The purity of mAbs was monitored by 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to Laemmli et al. (1970). mAbs purified by GART–Sepharose consist of IgG only, whereas mAbs purified by DEAE Affi-Gel Blue were occasionally contaminated by a small amount of albumin and transferrin. The apparent titers of mAbs were determined by adding a serial dilution of mAb (less than 0.3 nM) to 1 nM $^{125}$I-$\alpha$BGT-labeled receptor, and the resultant complexes were precipitated by GART (Lindstrom et al., 1981). Antibody titers were expressed in moles of $^{125}$I-$\alpha$BGT binding sites bound per liter of mAb solution ($\alpha$BGT binds to the two acetylcholine binding sites on each receptor monomer).

*Fab Fragments.* Purified mAb (1 mg in 1 mL) was dialyzed overnight at 4 °C against PBS–$N_3$ containing 25 mM 2-mercaptoethanol and 1 mM EDTA at pH 8.0. Papain (10 $\mu$g, i.e., a 100:1 weight ratio) was added to the mixture and incubated for 4 h at 37 °C with constant agitation. Iodoacetamide (15 $\mu$L of 1 M in 0.1 M sodium phosphate, pH 7.5) was then added and incubated for a further 15 min at 37 °C. The digest was dialyzed extensively against 10 mM sodium phosphate buffer, pH 7.5, and then applied to 5 mL of DE-52 (Whatman) equilibrated with the same buffer. The column was eluted with 3 × 1 mL of 10 mM sodium phosphate, pH 7.5, followed by a linear gradient of 10 mM sodium phosphate and 0.1 M sodium phosphate plus 0.5 NaCl at pH 7.5. Fab peaks were pooled, dialyzed, and concentrated before the purity and titer were determined as above.

*Receptor Purification.* Electric organs dissected from live *Torpedo californica* (Pacific Biomarine, Venice, CA) were used fresh or stored at −70 °C. Frozen tissue (200–300 g) was broken into small chunks and blended with 1 volume of buffer conta ig 0.4 M NaCl, 5 mM EDTA, 5 mM $NaN_3$, and 1 mM PMSF, pH 7.5, in a 1-gal Waring blender for 2 min at maximum speed. All operations were carried out at 4 °C unless otherwise stated. The homogenate was further homogenized in a Brinkmann polytron at maximum setting for 15 s at 0 °C followed by spinning at 5000 rpm for 15 min in a Beckman 21B centrifuge using a JA10 rotor. The supernatant was passed through four layers of gauze, and the filtrate was ultracentrifuged at 16000 rpm for 2 h in a Beckman type 19 rotor. The pellet was then suspended in PBS–$N_3$ with 1 mM EDTA with 5 strokes of a motor-driven Teflon homogenizer. This fraction was extracted with 2% sodium cholate–5 mg/mL soybean lipid in PBS–$N_3$, and the solubilized receptor was purified by affinity chromatography on toxin–agarose as previously described (Lindstrom et al., 1980).

Alternatively, receptor was purified by pH 11 extraction of native membranes. To obtain pH 11 extracted membrane fragments, the resuspended pellet after centrifugation in the type 19 rotor was diluted to about 10 mg/mL protein with PBS–$N_3$ and adjusted to 32% w/w sucrose in PBS–$N_3$ by adding a 66% w/w sucrose solution. The suspension (12 mL) was layered onto 12 mL of 36% w/w sucrose in PBS–$N_3$ and then overlaid with 1 mL of PBS–$N_3$. The step gradient was then spun at 45000 rpm for 1 h in a Beckman Ti50.2 rotor, which resulted in a pellet at the bottom of the tube, a midband at the interface of 32% and 36% sucrose solutions, and a top band situated at the PBS–$N_3$–32% sucrose solution interface. The midbands contained the highest specific activity (1000–1500 nmol of $^{125}$I-$\alpha$BGT binding sites per g of protein) and were removed and diluted with 3 volumes of cold distilled water. The pH of the suspension was adjusted to 11 with 0.2 M NaOH (Neubig & Cohen, 1979). After 15 min at room temperature, the mixture was spun at 30000 rpm for 30 min in a Beckman Ti50.2 rotor. The pellet was rinsed with buffer, resuspended, diluted in PBS–$N_3$, and respun. The pellet was finally resuspended in PBS–$N_3$ containing 5% sucrose. The specific activity of the pH 11 extracted vesicles thus obtained was about 4000 nmol of $^{125}$I-$\alpha$BGT binding sites per g of protein, whereas the specific activity of affinity-purified receptor was usually 7000–8000 nmol of $^{125}$I-$\alpha$BGT binding sites per g of protein. The pH 11 extracted vesicles were dissolved in 2% sodium cholate–5 mg/mL soybean lipid in PBS–$N_3$ at a protein concentration of 2.5 mg/mL such that the ratio of cholate:lipid:protein was 4:1:0.5 (Wu & Raftery, 1981; Anholt et al., 1981; Criado et al., 1982). The solution was spun at 40000 rpm for 20 min in a Beckman type 50 rotor. Receptor concentrations were expressed as $^{125}$I-$\alpha$BGT binding site concentrations determined by indirect immunoprecipitation of $^{125}$I-$\alpha$BGT-labeled receptor (Lindstrom et al., 1981).

*Reconstitution.* Reconstitution of receptors into lipid vesicles was carried out according to Anholt et al. (1982) with modifications. The reconstitution mixture contained solubilized receptor at 0.4 $\mu$M, varying amounts of purified mAbs or Fabs, 1.2% sodium cholate, and 40 mg/mL lipid (80% soybean lipid, 20% cholesterol) in PBS–$N_3$, pH 7.5. Incubations in the absence of mAbs or Fabs or in the presence of comparable concentrations of similarly purified rat IgG were used as controls. The mixture was incubated for 1 h at room temperature with gentle shaking before the sodium cholate was removed at 4 °C by dialysis against PBS–$N_3$ overnight followed by dialysis overnight against flux buffer (145 mM sucrose, 10 mM sodium phosphate, and 10 mM $NaN_3$, pH 7.5). The preparation was then frozen at −20 °C and thawed at ambient temperature. The purpose of the freeze–thaw cycle was to increase the size of the vesicles (Anholt et al., 1982). Receptor concentration in reconstituted vesicles was determined either in PBS–$N_3$, to measure the amount of receptor oriented right-side out, or in 0.5% Triton X-100 and PBS–$N_3$, to give the total amount of receptor. In a typical control preparation, 50–60% of the receptor was oriented right-side out.

*$^{22}Na^+$ Uptake Assay.* Uptake of $^{22}Na^+$ (New England Nuclear) into reconstituted vesicles during a 10-s incubation period in the presence of carbamylcholine was assayed as

Table I: Properties of Monoclonal Antibodies 148 and 168[a]

| property | mAb 148 | mAb 168 |
|---|---|---|
| immunized animal | Lewis rat | Lewis rat |
| immunogen | *Torpedo* $\gamma$ and SDS-denatured receptor | *Torpedo* $\delta$ and SDS-denatured receptor |
| myeloma cell line | S 194 | S 194 |
| Ig class | $IgG_2$ | $IgG_1$ |
| affinity, $K_D$ (Scatchard plot) (M) | $2.7 \times 10^{-9}$ | $1.8 \times 10^{-8}$ |
| subunit specificity | $\beta$ | $\gamma$ |
| species specificity | *Torpedo*, frog, rat, mouse, human | *Torpedo*, *electrophorus*, frog, rat, mouse, human |
| transmembrane orientation of determinant | cytoplasmic | cytoplasmic |
| interaction of mAb between receptors | 1 mAb bound per receptor, cross-links receptor | 1 mAb bound per receptor, cross-links receptor |
| generation of Fab–papain sensitivity | low | high |

[a] These hybridomas were initially prepared by Tzartos and co-workers (unpublished results). Their subunit specificity was first reported by Gullick & Lindstrom (1983), who used immune precipitation of $^{125}$I-subunits, and their transmembrane orientation was first reported by Sargent et al. (1984), who used histochemical methods on frog muscle. Subunit specificity has been confirmed by using ELISA assays with purified subunits, and transmembrane orientation has been confirmed by immune precipitation and ELISA assays using *Torpedo* receptor rich membrane vesicles (S. Hochschwender, L. Langeberg, D. Schneider, and J. M. Lindstrom, unpublished results).

previously described (Lindstrom et al., 1980). Background values were determined by substituting distilled water for the agonist solution in the assay. A typical reaction mixture contained 40 $\mu$L of reconstituted vesicles in flux buffer, 5 $\mu$L of $^{22}Na^+$ solution (0.2 mCi/mL), and 5 $\mu$L of 1 mM carbamylcholine solution or distilled water. The $Na^+$ content was 26 mequiv as determined by flame photometry. Counting efficiency was 85%.

*Kinetics of $^{125}$I-$\alpha$BGT Binding.* Rates of $^{125}$I-$\alpha$BGT binding were measured in flux buffer containing about $12 \times 10^{-9}$ M $^{125}$I-$\alpha$BGT and $3 \times 10^{-9}$ M $\alpha$BGT binding sites, with or without carbamylcholine. At time points, 25-$\mu$L aliquots were removed and spotted onto disks of dry DE-81 paper (Whatman) and then washed for 30 min in a buffer containing 0.1% Triton X-100, 50 mM NaCl, 10 mM sodium phosphate, and 2 mM carbamylcholine at pH 7.5. The wash solution was drained off carefully and replaced with 1 L of the same buffer containing no carbamylcholine. The washing was repeated 2 more times for 30 min. The DE-81 disks were blotted dry and counted in a $\gamma$ counter. Blanks (1000–2000 cpm) obtained by application of $^{125}$I-$\alpha$BGT to the disks in the absence of receptor were the same as blanks obtained following a 1-h preincubation of the receptor with 1 $\mu$M unlabeled $\alpha$BGT. Association of $^{125}$I-$\alpha$BGT with receptor was analyzed according to this second-order rate equation (Weiland et al., 1976):

$$\ln \frac{[T_0] - [RT]}{[R_0] - [RT]} - \ln \frac{[T_0]}{[R_0]} = ([T_0] - [R_0])k_t t$$

where $[T_0]$ and $[R_0]$ are the initial $^{125}$I-$\alpha$BGT and $\alpha$BGT binding site concentrations, respectively, [RT] is the concentration of the $^{125}$I-$\alpha$BGT–receptor complex at time $t$, and $k_t$ is the bimolecular association rate constant.

## Results

*Properties of mAbs.* Sixty-two affinity-purified mAbs were tested for effects on receptor functions by using receptor reconstituted into vesicles. Most of these mAbs were derived from immunizations with purified subunits or denatured receptor and, therefore, were directed at the cytoplasmic surface of the receptor (Froehner et al., 1983; Sargent et al., 1984; Anderson et al., 1983). Only two of these mAbs, mAb 148 and mAb 168, inhibited receptor function. The observation that most mAbs bound to receptor do not alter its function suggests that the sites to which these two mAbs bind are unusually important to receptor function. Some of the properties of mAbs 148 and 168 are shown in Table I. mAb 148 is specific for the $\beta$ subunit whereas mAb 168 is specific for the $\gamma$ subunit (Gullick & Lindstrom, 1983).

The transmembrane orientation of the mAb binding sites on receptors from *Torpedo* was determined. In receptor-rich membrane vesicle preparations, about 90% of the vesicles are sealed right-side out, and saponin can be used to permeabilize these sealed vesicles (Froehner et al., 1983). The ability of antibodies to bind to native and permeabilized vesicles was tested (Table II). These results suggest both mAb 148 and mAb 168 bind to determinants on the cytoplasmic surface of receptors which are accessible to the mAbs only after the native membrane vesicles are made permeable to antibodies by saponin. Similar results were obtained by Sargent et al. (1984), who observed that binding of mAbs 148 and 168 to frog muscle end plates could be detected only after the muscles were permeablized with saponin.

The ability of mAbs to bind to and cross-link solubilized receptors was studied by using sucrose gradients as initially described by Conti-Tronconi et al. (1981). Purified *Torpedo* receptors exist as dimers and monomers (Figure 1A). mAb 35 and mAb 5 were used as reference standards (Figure 1B,C). mAb 35 binds to the main immunogenic region on the extracellular surface of $\alpha$ subunits. In mAb 35 excess, two mAbs bind per receptor, and in receptor excess, the mAbs can cross-link receptors (Conti-Tronconi et al., 1981). By contrast, mAb 5 binds to the cytoplasmic surface of $\alpha$ subunits (unpublished results), and at any ratio of mAb 5 per receptor,

Table II: Effect of Permeabilization by Saponin on Binding of mAbs to Native Receptor-Rich Vesicles[a]

| | 3% saponin | | 0% saponin | |
|---|---|---|---|---|
| | cpm blank ± SD | % | cpm blank ± SD | % |
| mAb 148 | $(2.47 \pm 0.034) \times 10^5$ | 87 | $(3.85 \pm 0.42) \times 10^3$ | 29 |
| mAb 168 | $(1.95 \pm 0.066) \times 10^5$ | 68 | $(1.64 \pm 0.06) \times 10^3$ | 14 |
| rat antireceptor serum | $(2.84 \pm 0.006) \times 10^5$ | 100 | $(8.95 \pm 0.30) \times 10^3$ | 100 |

[a] Native membrane vesicles with 1.1 $\mu$M (3% saponin experiment) or 0.31 $\mu$M (0% saponin experiment) receptor were labeled with a 2-fold molar excess of $^{125}$I-$\alpha$BGT in PBS–$N_3$, pH 7.5. To 1-mL aliquots of this solution, mAbs were added to a final concentration of 11 $\mu$M (with saponin) or 3.1 $\mu$M (without saponin). As a control, rat antireceptor serum (5 $\mu$L of $1 \times 10^{-4}$ M) was added. The reaction mixtures were incubated overnight at 4 °C, followed by centrifugation in a Beckman airfuge. The supernatants were removed, and the pellets were dissolved in 1 mL of 0.5% Triton X-100 and PBS–$N_3$. The labeled receptors which were bound by antibody were determined by immunoprecipitation with GART in the presence of 5 $\mu$L of normal rat serum as carrier.

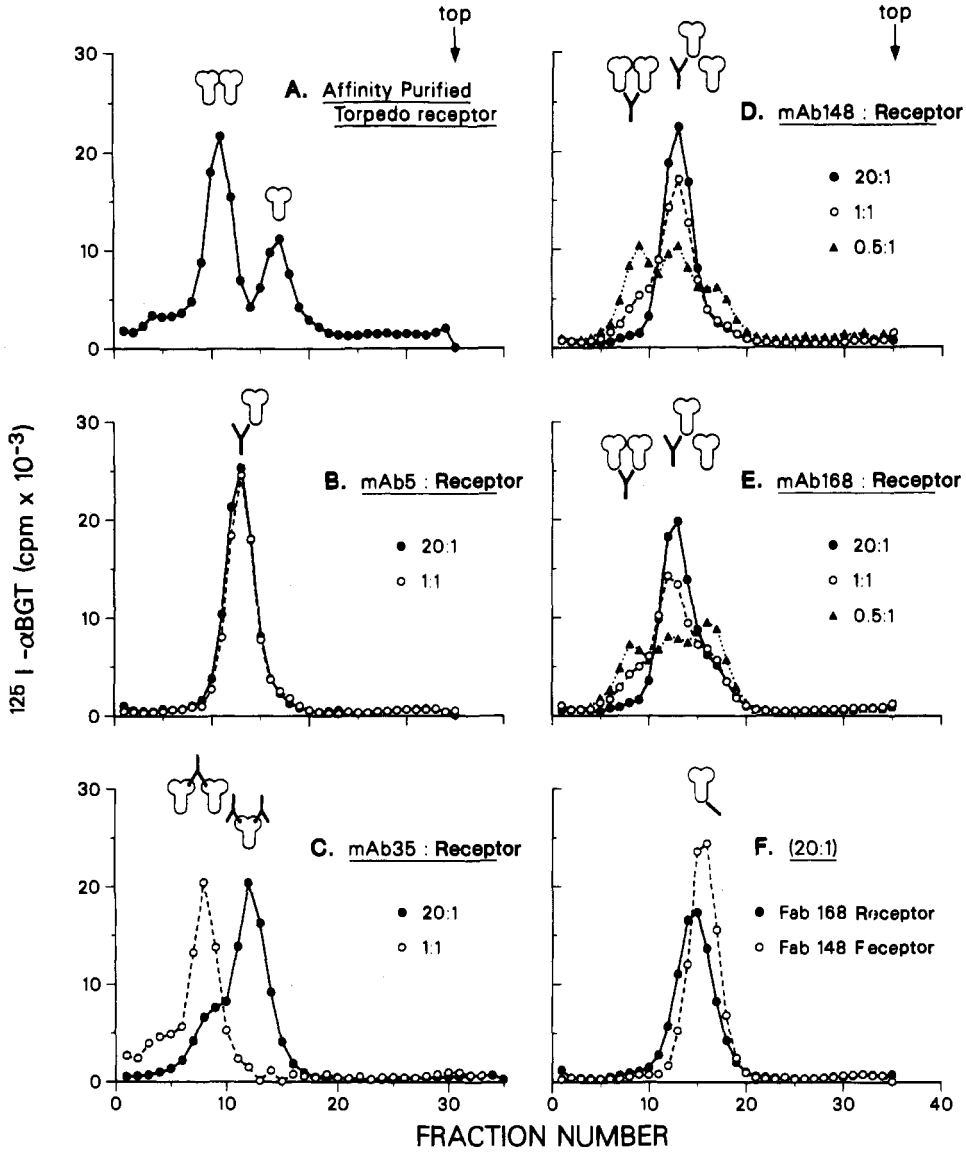


FIGURE 1: Resolution of mAb–receptor complexes by sucrose gradient centrifugation. Receptor monomers labeled with $^{125}$I-αBGT were isolated by centrifugation on preparative sucrose density gradients (Conti-Tronconi et al., 1981). Complexes between mAbs (at various concentrations) and $^{125}$I-αBGT-labeled monomers ($1 \times 10^{-8}$ M) were made by overnight incubation at 4 °C in 0.5% Triton X-100, and PBS–$N_3$. Aliquots of 0.1 mL were layered on 4.9 mL of 5–20% linear sucrose gradients in the same buffer as above. The gradients were spun in a Beckman VTi65 rotor at 61 500 rpm for 67 min at 4 °C. $^{125}$I-αBGT was measured in 135-μL (14-drop) fractions. The presence of bound mAb in the peaks was also demonstrated by immunoprecipitation with GART (data not shown). The symbols over the peaks indicate the type of complexes thought to account for the observed peaks. (A) Resolution of purified receptor into dimers and monomers. (B) Monomers plus mAb 5: mAb:receptor ratio = 20 (●); mAb:receptor ratio = 1 (○). (C) Monomers plus mAb 35; mAb:receptor ratio = 20 (●); mAb:receptor ratio = 1 (○). (D) Monomers plus mAb 148: mAb:receptor ratio = 20 (●); mAb:receptor ratio = 1 (○); mAb:receptor ratio = 0.5 (▲). (E) Monomers plus mAb 168: mAb:receptor ratio = 40 (●); mAb:receptor ratio = 1 (○); mAb:receptor ratio = 0.5 (▲). (F) Monomers plus Fab fragments: Fab 168:receptor ratio = 20 (●); Fab 148:receptor ratio = 20 (○).

complexes of only one mAb and one receptor are formed, presumably because mAb 5 cross-links the two α subunits within a receptor monomer (Conti-Tronconi et al., 1981). In antibody excess, mAb 148 and mAb 168 formed complexes of the size expected of one mAb and one receptor, whereas in receptor excess, complexes characteristic of one mAb and two receptors were formed (Figure 1D,E). Thus, as expected due to the presence of one β and one γ subunits in each monomer, both mAb 148 and mAb 168 bind to only one determinant on each monomer, and both mAbs can cross-link receptor monomers.

*Reconstitution.* Initially, affinity-purified receptors were used for reconstitution (Anholt et al., 1982). However, cholate–lipid extracts of pH 11 extracted receptor-rich membrane vesicles were nearly as pure. Carbamylcholine-induced $^{22}Na^+$ influx in vesicles reconstituted from soluble extracts from pH 11 vesicles was linearly proportional to the receptor concentration (Figure 2). Since making pH 11 vesicles extracts was faster, this approach was used for subsequent studies with mAbs 148 and 168.

To allow for mAbs that bind to cytoplasmic determinants such as mAb 148 and mAb 168 mAbs were added to the reconstitution mixture before dialysis. Thus, in the resulting liposomal suspension, mAb was present both in the extravesicular medium and inside the vesicle lumen. This was verified by immunoprecipitation of receptors solubilized from vesicles freed from extravesicular mAb by centrifugation. mAbs could also be introduced into vesicles by freezing and thawing the vesicles in the presence of mAb, as was demonstrated with $^{125}$I-mAbs (data not shown).

*Effect of mAbs on Agonist-Induced $^{22}Na^+$ Uptake.* Inhibition of receptor function by mAbs 148 and 168 was ob-

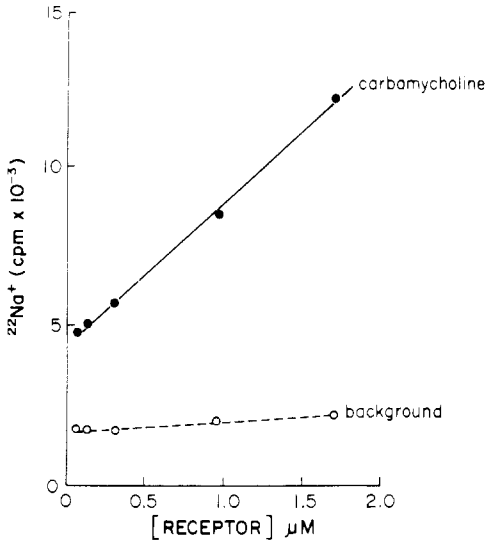


FIGURE 2: $^{22}Na^+$ uptake in reconstituted vesicles at various receptor concentrations. Various amounts of receptor extracted from pH 11 treated vesicles were reconstituted into liposomes as described under Materials and Methods. $^{22}Na^+$ uptake in the presence of 0.1 mM carbamylcholine was assayed as described under Materials and Methods. Receptor concentrations were expressed as micromolar concentrations of αBGT binding sites.

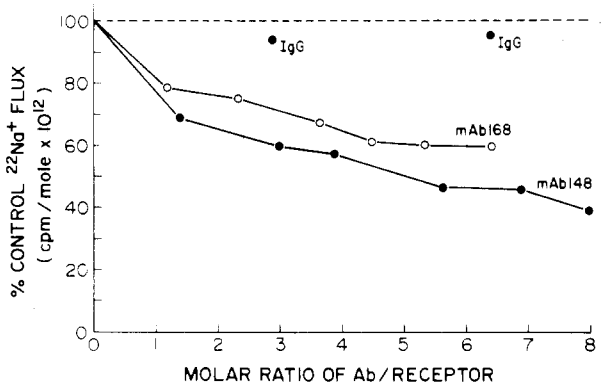


FIGURE 3: Effect of mAbs 148 and 168 on carbamylcholine-induced $^{22}Na^+$ uptake by vesicles containing purified receptor. Purified receptors were reconstituted in the presence of various amount of mAbs. Molar concentrations of mAbs were obtained by dividing the grams of IgG per liter (1.41 $A_{280}$ = 1 g/L) by 150000 and by dividing the concentrations of receptor in moles per liter of $^{125}I$-αBGT binding sites by 2. The amplitudes of $^{22}Na^+$ uptake are expressed as cpm × $10^{12}$ per mol of external αBGT sites in the presence of 0.1 mM carbamylcholine after the background is subtracted. The receptor concentration was 0.567 ± 0.028 μM, and 30 μL was used in the assay. For the control, cpm/0.1 mM carbamylcholine = 20000 ± 317 and cpm/$H_2O$ = 4750 ± 397 ($N$ = 3).

served in many experiments. A typical experiment is described below. Figure 3 shows inhibition of carbamylcholine-induced $^{22}Na^+$ uptake into vesicles when receptor was reconstituted in the presence of different amounts of mAbs 148 and 168. As a control, purified normal rat IgG was shown to have no effect on $^{22}Na^+$ uptake in reconstituted vesicles. It is interesting to note that for both mAbs, the maximum inhibition was about 50% of control. mAb 148 seemed to inhibit $^{22}Na^+$ uptake more than did mAb 168. Figure 4 shows a dose–response curve for carbamylcholine-induced $^{22}Na^+$ uptake into vesicles in the presence of mAb 148 and mAb 168. Linear transformations of the straight-line portion of the slopes of the mAb 148 and mAb 168 dose–response curves (Figure 4, inset) suggest noncompetitive inhibition, which is expected of mAbs binding to cytoplasmic determinants.

To show that mAbs 148 and mAb 168 act by binding to

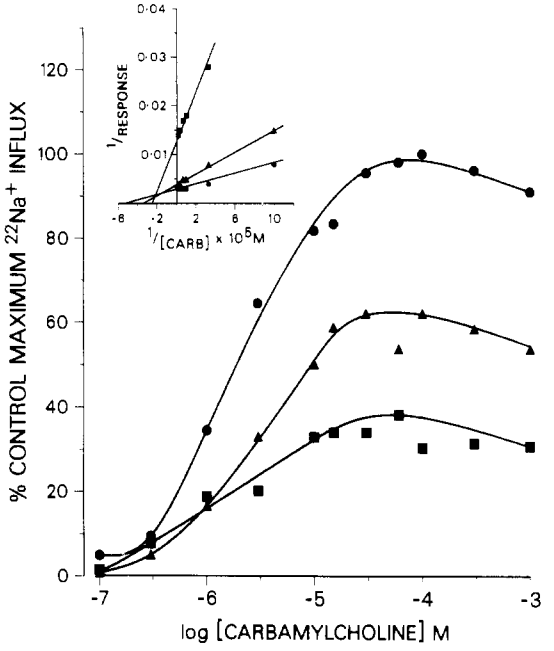


FIGURE 4: Dose dependence of receptor activation in reconstituted vesicles. Conditions used were those described under Materials and Methods and in the legend to Figure 3: (●) control; (▲) mAb 168:receptor ratio = 5.3; (■) mAb 148:receptor ratio = 8.0. External αBGT binding site concentrations were 0.50 μM for the control, 0.70 μM for mAb 168, and 0.53 μM for mAb 148, and 24 μL was used in the assay mixture in each case. The maximal response in the control was 5910 ± 563 cpm, and the background was 1260 ± 248 cpm ($N$ = 3). Inset: Double-reciprocal plot of the dose–response curves from $10^{-6}$ to 6.0 × $10^{-5}$ M carbamylcholine.

Table III: Effect of mAbs on Carbamylcholine-Induced $^{22}Na^+$ Uptake in Reconstituted Vesicles

| | mAb:receptor ratio | % control ($10^{-4}$ M carbamylcholine) ± SD[a] mAb added before reconstitution | mAb added after reconstitution | Fab added before reconstitution |
|---|---|---|---|---|
| mAb 168 | 5.3 | 60 ± 3 | 93 ± 5 | 72 ± 8 |
| mAb 148 | 8.0 | 31 ± 1 | 82 ± 9 | 95 ± 3 |
| IgG | | 95 ± 2 | 86 ± 9 | 95 ± 3 |

[a] The amplitudes of the response (in cpm × $10^{12}$ per mole of αBGT binding sites) for controls were 387.2 ± 46.9 for mAb added before reconstitution, 306.9 ± 18.7 for mAb added after reconstitution, and 854.2 ± 17.6 for Fab added before reconstitution. The concentration of purified IgG added as control was 7.2 μM or IgG:receptor ratio = 6.4.

cytoplasmic determinants, preformed vesicles were incubated with the mAbs. In this case, both carbamylcholine and mAb were present in the extravesicular medium. Under such circumstances, neither mAb 148 nor mAb 168 affected $^{22}Na^+$ flux. By contrast, when these mAbs were present on the cytoplasmic side and carbamylcholine was on the agonist site side, the ion flux was inhibited. These data are summarized in Table III. Furthermore, when mAbs were added to preformed reconstituted vesicles, a cycle of freezing and thawing permitted entry of the mAbs which could then block function (Data not shown).

*Effect on $^{125}I$-αBGT Binding.* In the absence of carbamylcholine and in $^{125}I$-αBGT excess, the kinetics of $^{125}I$-αBGT binding to both reconstituted vesicles and native membranes were biphasic, with an initial fast phase followed by a slow phase (Figure 5). These results are similar to those of Leprince et al. (1981). As shown in Figure 5, the rates of $^{125}I$-αBGT binding to receptor reconstituted in the presence of mAb 168 and mAb 148 were decreased. This effect was most

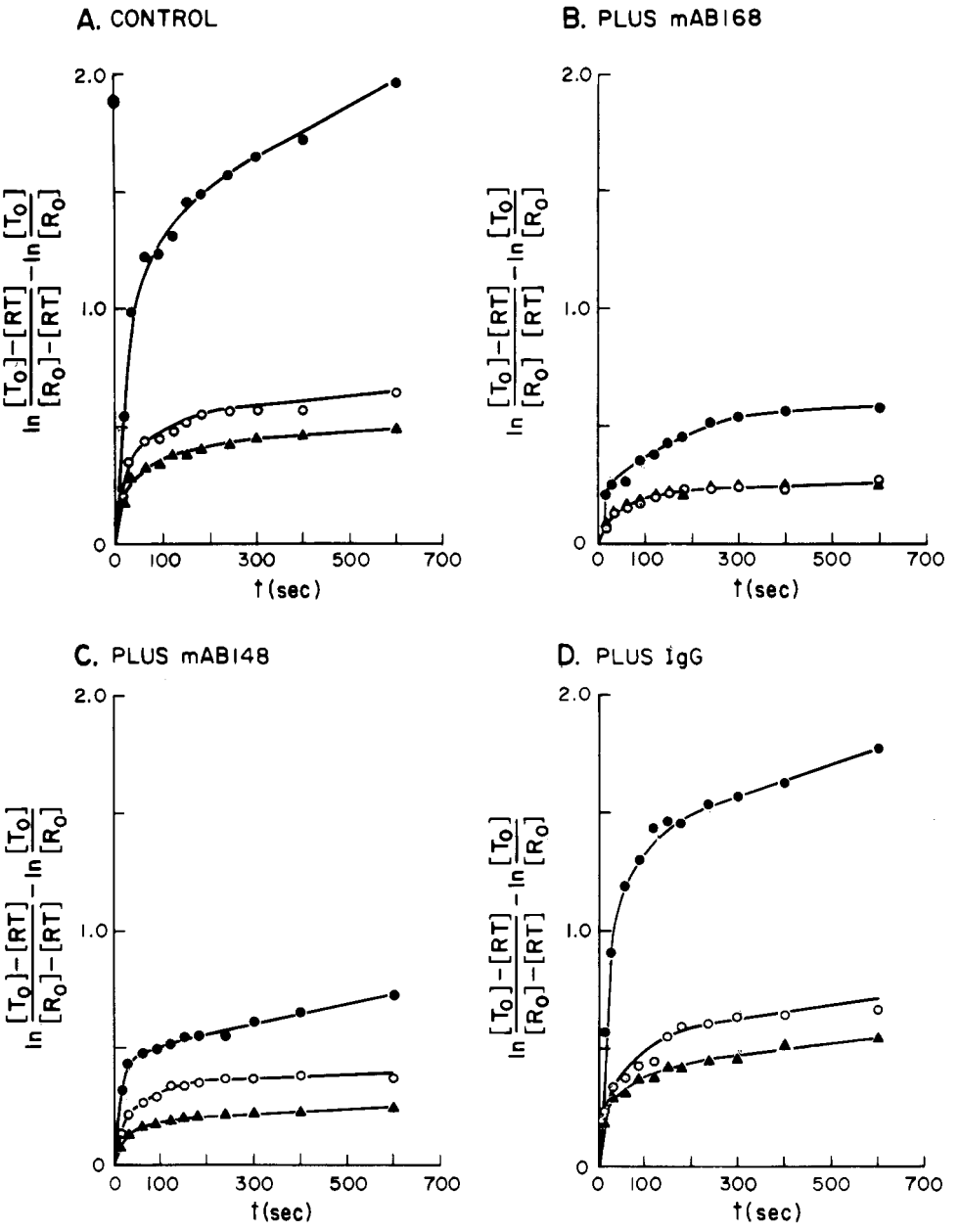


FIGURE 5: $^{125}$I-αBGT binding rates in the presence of mAbs. $^{125}$I-αBGT binding was carried out as described under Materials and Methods: (●) no carbamylcholine; (○) after 10-s exposure to 2 μM carbamylcholine; (▲) after 30-min exposure to 2 μM carbamylcholine. (A) Control, $[R_0] = 7.916 \times 10^{-9}$ M; (B) mAb 168:receptor ratio = 5.3, $[R_0] = 2.940 \times 10^{-9}$ M; (C) mAb 148:receptor ratio = 8.0, $[R_0] = 2.968 \times 10^{-9}$ M; (D) IgG:receptor ratio = 6.4, $[R_0] = 2.95 \times 10^{-9}$ M. $[T_0]$ was $12 \times 10^{-9}$ M in all cases.

pronounced with resting receptors (no carbomylcholine added) but was also evident with activated receptors (carbamylcholine added for 10 s) and with desensitized receptors (carbamylcholine added for 30 min). By contrast, vesicles reconstituted similarly in the presence of purified normal IgG bound $^{125}$I-αBGT like control vesicles (Figure 5D). In the presence of mAb 168, the curves for $^{125}$I-αBGT binding after pretreatment of the reconstituted vesicles with carbamylcholine for 10 s and 30 min were superimposable (Figure 5B). This suggests that mAb 168 may have facilitated desensitization. Although both mAbs had pronounced effects on the rate of $^{125}$I-αBGT binding to reconstituted receptor, neither mAb 168 nor mAb 148 effected $^{125}$I-αBGT binding at equilibrium, as shown in Table IV.

*Effect of mAbs on the Association of Receptor with Lipids during Reconstitution.* Gel exclusion chromatography with Sephacryl S-1000 was used for size analysis of vesicle preparations reconstituted with or without mAbs (Nozaki et al.,

Table IV: Effect of mAbs on $^{125}$I-αBGT Binding at Equilibrium

| | molar ratio mAb:receptor | $^{125}$I-αBGT–receptor (soluble) pptd (×10$^6$ M ± SD)[a] | $^{125}$I-αBGT–receptor in vesicles (×10$^9$ M ± SD) |
|---|---|---|---|
| mAb 168 | 5.30 | 0.74 ± 0.04 | 2.23 ± 0.42 |
| | 2.65 | 0.71 ± 0.05 | |
| | 1.32 | 0.76 ± 0.03 | |
| mAb 148 | 8.00 | 0.76 ± 0.01 | 2.17 ± 0.34 |
| | 4.00 | 0.75 ± 0.03 | |
| | 2.00 | 0.74 ± 0.03 | |
| control | | 0.75 ± 0.03 | 2.63 ± 0.23 |

[a] Purified receptor ($2.5 \pm 10^{-6}$ M) solubilized in 2% sodium cholate–5 mg/mL soybean lipid was incubated with various amounts of mAbs as indicated or without mAbs overnight at 4 °C. Excess $^{125}$I-αBGT (10 μM) was added and incubated at room temperature for 4 h. The $^{125}$I-αBGT–receptor complexes were then precipitated by adding GART. All experiments were done in triplicate. [b] Reconstituted vesicles with or without mAbs ($2.75 \times 10^{-9}$ M) were incubated with excess $^{125}$I-αBGT ($11 \times 10^{-9}$ M) overnight at 4 °C. The $^{125}$I-αBGT–receptor complexes were quantitated by DE-81 filters as described under Materials and Methods. All experiments were done in triplicate.

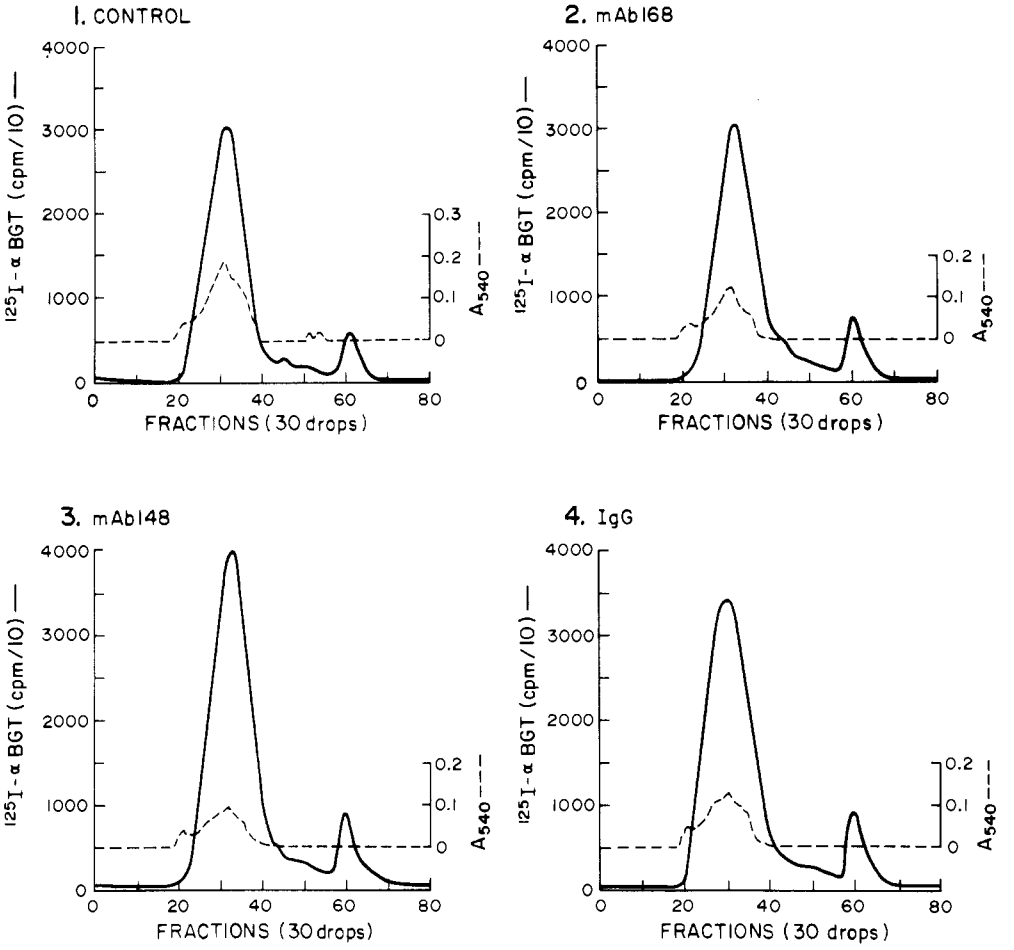


FIGURE 6: Sephacryl S-1000 elution profiles of vesicles reconstituted in the presence or absence of mAbs. Reconstituted vesicles containing 0.53–0.70 μM αBGT binding sites were labeled with $8 \times 10^{-9}$ M $^{125}$I-αBGT by incubating at room temperature for 3 h. After being adjusted to 8.6% w/v sucrose, 0.25 mL of the mixture was immediately applied to a Sephacryl S-1000 (Sigma) column (85 × 10.8 cm) equilibrated in PBS–$N_3$ with 145 mM sucrose. The column was eluted with the same buffer at room temperature with a pressure head of 17 cm$H_2O$ (about 2–4 mL/h). Fractions of 30 drops (about 0.72 mL) were collected and counted in a γ counter, and the optical densities at $A_{540}$ were measured. The column was packed at 50 cm$H_2O$, and the $V_0$ was determined to be 14.4 mL (i.e., 20 fractions). Prior to each run, the column was equilibrated with lipids to prevent adsorption during chromatography by passing 1 mL of reconstituted vesicles through and then washing with 1 column volume of elution buffer. The amounts of receptor used, in terms of $^{125}$I-αBGT binding sites, were the following: (1) control, 0.54 μM; (2) mAb 168, 0.53 μM; (3) mAb 148, 0.70 μM; (4) purified IgG, 0.60 μM.

1982). Each profile in Figure 6 showed that essentially all of the $^{125}$I-αBGT binding components coeluted with the majority of the turbidity (presumably vesicles). Radiotracer studies using [$^3$H]cholesterol and [$^{14}$C]dipalmitoylphosphatidylcholine indicated that these fractions contain the lipids used for reconstitution (data not shown) and presumably they are receptor-containing vesicles. Since all of the reconstituted vesicles were eluted as a symmetrical peak in fractions centered around 30–32, apparently a homogeneous population of vesicles in terms of size was formed in each case, and neither mAb effected the reconstitution process. The results obtained by Nozaki et al. (1982) suggest that these vesicles have an estimated diameter of about 200 nm.

$^{125}$I-αBGT trace-labeled vesicles, when centrifuged to equilibrium on a sucrose gradient, formed symmetrical peaks at about 15% w/w sucrose, whether in the presence or absence of mAbs, indicating that in each case a homogeneous population of vesicles similar in density was formed (data not shown). Radiotracer studies also showed that [$^3$H]cholesterol and [$^{14}$C]dipalmitoylphosphatidylcholine associated exclusively with these peaks (data not shown). No other $^{125}$I-αBGT binding components were detected. Again these results suggest mAbs have no effect on reconstitution of receptor into liposomes. It also seems that bound mAbs have no effect on the buoyancy of the liposomes.

*Effect of Fab Fragments on Receptor Function in Reconstituted Vesicles.* Purified receptors were also reconstituted in the presence of monovalent Fab fragments generated from mAbs by papain treatment. As shown in Table III, Fab 148 had no effect on $^{22}Na^+$ uptake in the reconstituted vesicles, whereas Fab 168 inhibited only 28% as compared to 40% inhibition observed with the intact molecule. $^{125}$I-αBGT binding studies also revealed that Fab fragments of neither mAb 148 nor mAb 168 effected the rate of $^{125}$I-αBGT binding to receptor reconstituted in the presence of these fragments (Figure 7). Bound mAbs and Fab fragments were demonstrated in reconstituted vesicles. In vesicles exposed to Fabs 148 and 168, 86% and 74%, respectively, of the receptors were shown to have Fabs bound by pelleting the vesicles to remove unbound Fabs and then solubilizing in Triton X-100 and measuring the fraction of receptors bound with Fabs by indirect immune precipitation, which compares favorably with values of 92% and 65% when intact mAbs were used in the same experiment. Thus, the lack of effect of Fab fragments is not due to lack of binding.

## Discussion

Here we used purified mAbs as probes to alter the function of purified receptors reconstituted into lipid vesicles. Previously, anti-receptor sera have been shown to inhibit ligand

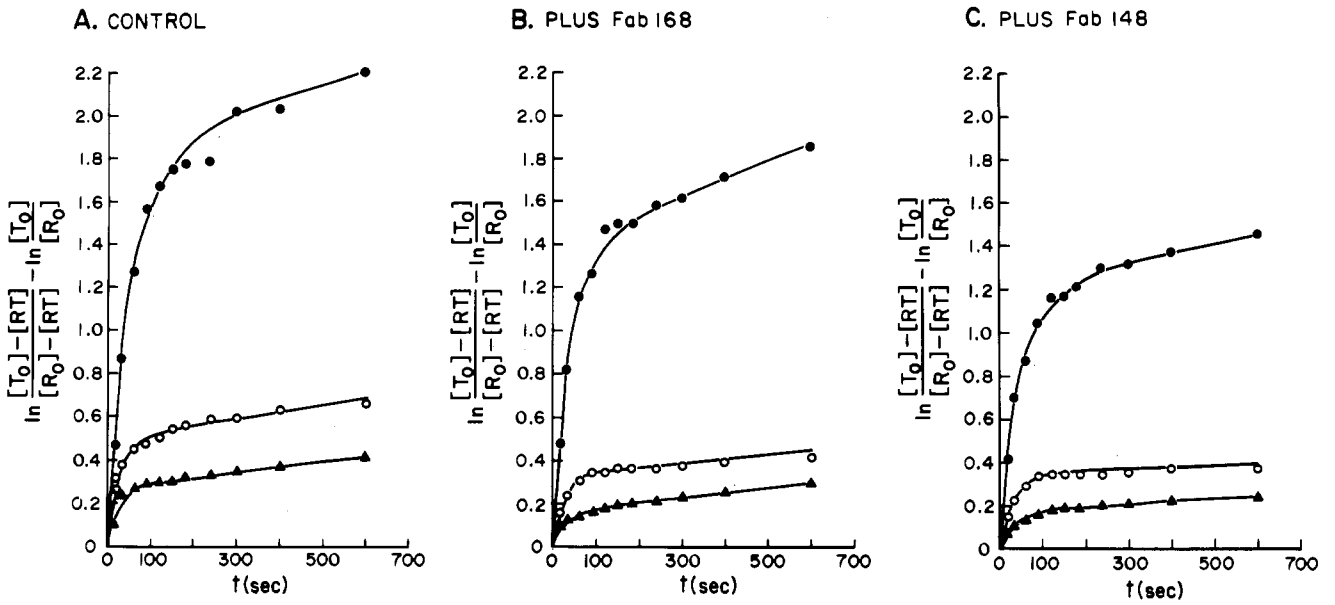


FIGURE 7: $^{125}$I-$\alpha$BGT binding rates in the presence of Fab fragments. The rate of $\alpha$BGT binding was carried out as described under Materials and Methods: (●) no carbamylcholine; (○) after 10-s exposure to 2 $\mu$M carbamylcholine; (▲) after 30-min exposure to 2 $\mu$M carbamylcholine. (A) control, $[R_0] = 2.898 \times 10^{-9}$ M; (B) Fab 168:receptor ratio = 5.3, $[R_0] = 2.916 \times 10^{-9}$ M; (C) Fab 148:receptor ratio = 8.0, $[R_0] = 2.960 \times 10^{-9}$ M. $[T_0]$ was $12 \times 10^{-9}$ M in (A–C).

binding and cation uptake in receptor-rich membranes (Mihovilovic & Martinez-Carrion, 1979; Fulpius et al., 1980; Desouki et al., 1981; Gonzalez-Ros et al., 1984). However, serum antibodies are polyclonal, and the acetylcholine receptor rich membranes contain many other proteins. Other groups have used mAbs for structure–function studies (Gomez et al., 1979; James et al., 1980; Mochly-Rosen & Fuchs, 1981; Lindstrom et al., 1981b; Watters & Maelicke, 1983; Gomez & Richman, 1983). With the exception of Lindstrom et al. (1981b, 1984), who reported inhibition of receptor function by a mAb specific for the extracellular determinants on the $\alpha$ and $\beta$ subunits and lack of inhibition of function by antibodies to the main immunogenic region, all the other groups have used mAbs directed at or near the ligand binding sites. Although mAbs specific for the acetylcholine binding sites are valuable in probing this functionally important region on the receptor molecule, toxins and affinity labeling reagents are available for these sites (Karlin, 1980), and it is especially interesting to determine the functional roles of subunits other than $\alpha$. Recent success in the study of the cDNA-directed synthesis of the functional receptor by Numa and co-workers (Mishina et al., 1984) has indicated that all four subunits are required for a normal response to acetylcholine, whereas only the $\alpha$ subunit is required for $\alpha$BGT binding. Furthermore, cytoplasmic determinants of the receptor have been shown to be phosphorylated by cAMP-dependent kinases (Huganir & Greengard, 1983), and it has been suggested that modifications of the cytoplasmic portion of the receptor might have significant regulatory roles in receptor functions (Changeux, 1981). Our laboratory has accumulated many mAbs directed toward different parts of the receptor molecule. Most of these have no obvious effect on receptor function (Lindstrom et al., 1981a).

We report here that 2 of 62 mAbs tested, mAbs 148 and 168, inhibit agonist-induced cation uptake. Their effect is not due to interference with the reconstitution process (Figure 6). These mAbs do not competitively inhibit carbamylcholine-induced receptor activation (Figure 4). These two mAbs are especially interesting because they are specific for cytoplasmic determinants on the $\beta$ (mAb 148) and $\gamma$ (mAb 168) subunits, suggesting that both $\beta$ and $\gamma$ subunits are also involved in the regulation of receptor function and this effect can be exerted from the cytoplasmic side. These mAbs affect the conformation of the receptor at sites distant from their binding sites because binding of these antibodies to the cytoplasmic surface of the receptor alters binding of $^{125}$I-$\alpha$BGT to the extracellular surface (Figure 5).

Even large excesses of mAbs 148 or 168 did not completely inhibit carbamylcholine-induced $^{22}Na^+$ influx into vesicles (Figure 3). Measurements of carbamylcholine-induced $^{22}Na^+$ uptake in reconstituted vesicles have low resolution because measurements of uptake made in tens of seconds are used to assay receptors whose channels open for milliseconds, which are subject to desensitization, and whose high flux rates can rapidly equilibrate the small internal volume of a reconstituted vesicle. A better system in terms of time resolution (microseconds) is single-channel measurements. In collaboration with Dr. M. Montal of UCSD, both mAb 168 and mAb 148 were found to inhibit single receptor channel activity measured by using receptor reconstituted in lipid bilayers on patch pipets (Y. Blatt, K. K. Wan, J. M. Lindstrom, and M. Montal, unpublished results). Furthermore, it was shown that mAb 168 partially inhibits end-plate currents when injected into the cytoplasm of *Xenopus* muscle in culture (Tamaki, Wan, Lindstrom and Kidokoro, unpublished results).

Since monovalent Fab fragments had no effect (mAb 148) or a much less pronounced (mAb 168) effect on receptor function than did intact bivalent mAbs, and since a large fraction of the receptors was bound by Fabs, indicating that the monovalent Fabs retained high affinity, the effect of the bivalent mAbs must have been due primarily to cross-linking of receptors (Figure 1). Binding of these mAbs to these cytoplasmic determinants probably disturbed the whole receptor conformation rather than just occluding the cytoplasmic lumen of the channel because these mAbs also slow the rate of $\alpha$BGT binding to the extracellular surface of $\alpha$ subunits (Figure 5). Since $\alpha$BGT derives its high affinity from multiple contacts with the receptor (Kistler et al., 1982), it apparently eventually overcomes the inhibition of the initial binding rate caused by mAbs, which would explain the lack of effect of mAbs on $^{125}$I-$\alpha$BGT binding at equilibrium. Inhibition of receptor function by mAbs is specific to the sites of binding of these

mAbs because other mAbs which cross-link receptors, for example, mAbs to the main immunogenic region, do not block function (Lindstrom et al., 1981b, 1984). Thus, mapping the binding sites for these mAbs on the primary structures of the subunits should prove interesting and, along with detailed electrophysiological studies, may permit precise interpretation of the mechanisms by which mAb binding to these receptor substructures inhibits receptor function.

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**Registry No.** Na, 7440-23-5; carbamylcholine, 462-58-8.

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# Comparative Study of Glutamine Synthetase Bound Lanthanide(III) Ions Using NMR Relaxation and Lanthanide(III) Luminescence Techniques†

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*Received August 7, 1984*

ABSTRACT: Changes in the intrinsic fluorescence intensity of glutamine synthetase induced by lanthanide(III) ion binding demonstrate the existence of three types of sites for these ions. The sites are populated sequentially during titrations of the enzyme, and the first two have a stoichiometry of 1 per enzyme subunit. The number of water molecules coordinated to Eu(III) bound to the first site was determined by luminescence lifetime techniques to be 4.1 ± 0.5. The hydration of Gd(III) bound to the same site was studied by magnetic field dependent water proton longitudinal relaxation rate measurements, and by water proton and deuteron relaxation measurements of one sample at single magnetic fields. The magnetic resonance techniques also yield a value of 4 for the hydration number.

The number of water molecules directly coordinated to an enzyme-bound metal ion must often be determined in order to assess the role of the metal ion in substrate binding and catalysis. Several methods for determining this parameter have therefore been developed, including water proton and deuteron NMR relaxation techniques (Burton et al., 1979), detection of $^{17}O$ superhyperfine coupling to paramagnetic metal ions using electron paramagnetic resonance (EPR)[1] (Reed & Leyh, 1980), luminescence lifetime measurements of Eu(III) and Tb(III) (Horrocks & Sudnick, 1981), and, most recently, EPR spin-echo envelope modulation measurements (Peisach et al., 1984).

The most frequently applied method for studying paramagnetic metal ions such as Mn(II) and Gd(III) is the NMR technique of measuring water proton relaxation rates (PRR) as a function of magnetic field and/or temperature. In general, data analysis is carried out in terms of the Solomon–Bloembergen–Morgan (SBM) equations (Solomon, 1955; Bloembergen & Morgan, 1961) which were developed to account for the relaxation behavior of aqueous solutions of paramagnetic metal ions. The reliability of hydration numbers determined by this type of analysis is subject to some well-founded concern stemming from two main sources. First, since the SBM theory was derived for aqueous ions, it may not be applicable to macromolecular systems. Second, since data analysis often involves the use of a multiparameter fitting procedure, the results are subject to the well-known ambiguities inherent in this method of analysis. It would be useful to study systems of known hydration number in order to assess the applicability of the theory to macromolecular systems.

Some members of the series of lanthanide(III) ions possess spectroscopic properties which allow determinations of the metal ion hydration number by more than one technique. In particular, measurements of the lifetime of the luminescent $^5D_0$ state of Eu(III) in $H_2O$ and $D_2O$ provide a direct and accurate measure of metal ion hydration (Horrocks & Sudnick, 1979, 1981). Gd(III), which has an $^8S_{7/2}$ ground state, makes an ideal probe for water proton and deuteron relaxation studies (Reuben, 1971). Moreover, the nearly identical sizes and chemical properties of these two ions make direct comparison of experiments valid.

Glutamine synthetase from *Escherichia coli* is a well-characterized enzyme which has been the subject of many investigations using metal ions as spectroscopic probes. The active form of the enzyme has 12 identical subunits, each of molecular weight 50 000 (Ginsburg, 1972). Each subunit has two essential divalent metal ion binding sites, designated $n_1$ and $n_2$, in the catalytically active region and a third set of sites thought to stabilize the catalytically active enzyme structure. For the past several years, one of our laboratories has been investigating structure–function relationships in this allosterically regulated enzyme. Understanding the role of divalent cations in the catalytic mechanism has been one of the primary goals of this work (Villafranca & Balakrishnan, 1979).

Water proton relaxation measurements have been used to study changes in the environment of Mn(II) at the $n_1$ site, which is near the glutamate binding site, upon substrate, product, and inhibitor binding (Villafranca et al., 1976a,b; Villafranca & Wedler, 1974). Similar studies of the $n_2$ site, which is involved in nucleotide binding, have also been carried out (Ransom, 1984), and the distance relationship between these metal ion sites has recently been investigated by EPR spectroscopy (Gibbs et al., 1984). In one of the early applications of lanthanide(III) ion probes of enzymes, Wedler & D'Auroa (1974) used changes in the absorption spectrum of Nd(III) in a qualitative study of substrate interactions with glutamine synthetase. Thus, with lanthanide(III) ions as probes, glutamine synthetase provides a system in which results

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[1] Abbreviations: SBM, Solomon–Bloembergen–Morgan; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EPR, electron paramagnetic resonance; PRR, proton relaxation rate(s); EDTA, ethylenediaminetetraacetic acid.

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