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MASS-SCREENING OF CURARIMIMETIC NEUROTOXIN ANTAGONISTS

ANNUAL REPORT

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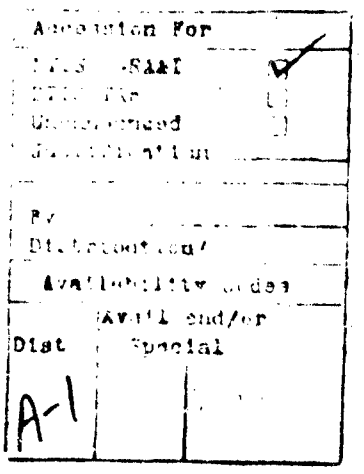

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A solid phase assay for ligands of curarimimetic toxin binding sites was developed. Its principle is the autoradiographic detection of the displacement of ^{125}I-alpha-bungarotoxin from Torpedo californica acetylcholine receptor. To permit analysis of large numbers of data, receptor was immobilized to multiwell titer plates. Several parameters were investigated to optimize the procedure, among them type of plate and plastic; nature of detergent used for receptor solubilization; and treatment to increase receptor adsorption to the wells. The final protocol involves cholate as a receptor extractant and flexible PVC plates coated with polyethyleneimine for maximal receptor adsorption. An autoradiographic procedure utilizing a special exposure cassette was devised. The possibility of an ELISA version of the assay was explored but a major goal, receptor adsorption in amounts sufficient for colorimetric detection, has not been reached.

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STATEMENT OF PROBLEM

As outlined in the First Annual Report, the goal of this contract is to develop methods that will eventually lead to the identification of therapeutically useful antagonists of curarimimetic neurotoxins, a group of snake venom proteins which block the nicotinic acetylcholine receptor in the neuromuscular junction thereby causing paralysis. The great majority of neurotoxin antagonists, i.e. substances that in vitro compete with or displace the neurotoxin, are likely to be receptor antagonists themselves, and consequently of little therapeutic value. It is therefore anticipated that the search for the relatively rare physiologically inert toxin antagonists will require a major effort which could be greatly helped by the development of labor-saving mass-screening methodologies.

BACKGROUND

A simple way of screening potential ligands for a specific receptor site is to measure their potency in displacing a known radioligand from that site. In this fashion the pharmacological profile of a receptor can be established. In studies of cholinergic receptors in the CNS, inhibition of [^{3}H]-acetylcholine binding to brain membranes by appropriate ligands has been used to identify a binding site as either nicotinic or muscarinic (Schwartz et al., 1982). Radioreceptor assays have been used to quantitate known receptor-specific drugs (Enna, 1985) as well as to identify and develop novel compounds with desired receptor binding properties (Snyder, 1983; Evans et al., 1986). This approach has not been used in the search for acetylcholine receptor toxin antidotes, although data describing the potency of various drugs in neurotoxin competition assays are scattered throughout the literature on nicotinic receptors (see e.g. Weber and Changeux, 1974; Schmidt and Raftery, 1975; Neubig and Cohen, 1979; Blanchard et al., 1979). In all of these studies the goal was to obtain information on the nicotinic nature of the binding site; or to quantitatively determine ligand affinities; or to study the phenomenon of desensitization. Consequently analysis of a relatively small number of ligands sufficed, and it was possible to employ various toxin binding assays without regard to their speed and simplicity.

RATIONALE

The basic assumption on which this research project rests is that the neurotoxin binding site in the nicotinic acetylcholine receptor differs sufficiently from the acetylcholine binding site that drugs can be found or designed which discriminate between them. This expectation

was borne in an investigation performed during the first contract year. Basic amino acids and in particular small basic proteins were shown to block toxin binding without impairing the binding of the agonist acetylcholine to the Torpedo electric tissue receptor. What remains to be shown is that such ligands act as physiologically inert toxin antagonists in preparations in which not only binding but ion transport is measured.

First however a mass screening procedure for the identification of toxin antagonists has to be developed. The goal here is to combine existing toxin binding assays with technologies adapted from hybridoma screening. During the first contract year I was able to show that the standard DEAE-cellulose assay for neurotoxin binding can be employed in an autoradiographic version, thereby greatly reducing the number of manipulations without sacrificing accuracy. The second year was primarily devoted to the development of a solid-phase assay as a prerequisite for complete automation. This goal has been reached ; a quick and reliable autoradiographic solid-phase assay is now available.

METHODS AND MATERIALS

(1) Ligand preparation:

During the course of the second contract year several batches of ^{125}I-alpha-bungarotoxin were prepared as described previously (Schmidt, 1987). Again, specific activities ranged from ca 1000 cpm/femtomole to 150 cpm/femtomole, depending on batch and age, for the monoiodinated derivative, and about twice as high for the diiodinated preparation. Alpha-bungarotoxin was conjugated to fetal intestinal alkaline phosphatase according to Ey et al. (1978). The alpha toxin of Naja naja siamensis was purified from freeze-dried venom (Miami Serpentarium) by chromatography on carboxymethyl-cellulose and Sephadex G-50. [^{3}H]-acetylcholine was synthesized as outlined before.

(2) Receptor preparation and assays:

Receptor-rich T.ocds californica electric tissue membranes were prepared essentially as described previously. For most applications in which solubilized receptor was required, concentrated membrane samples were diluted into 1% sodium cholate, 10 mM sodium phosphate pH 7.4. Receptor was quantitated in membranes and solution, using the DEAE-cellulose disk assay as described previously.

(3) Preparation of antibodies:

Anti Torpedo receptor antisera and monospecific anti-receptor antibodies were prepared as described

previously (First Annual Report). Purification of the monoclonal antibody mAb35, which recognizes the "main immunogenic region" of the alpha subunit of the Torpedo receptor was performed using either immobilized receptor or immobilized goat anti-rat immunoglobulin antiserum. The former resin was prepared by specifically adsorbing Torpedo receptor onto a resin containing alpha-bungarotoxin followed by covalent coupling with the cross-linking agent dimethyl pimelimidate (Pierce; for details see previous report); the latter by reacting 2 ml of a high-titer serum (PelFreez: 18.3 mg of anti-rat immunoglobulin antibody per ml of goat serum) with 10 ml of cyanogen-activated Sepharose 4B-CL (Pharmacia).

Monospecific anti-alpha-bungarotoxin antibodies were similarly obtained from an anti toxin serum by affinity chromatography on resin-coupled antigen.

Secondary antibodies: Goat anti-rabbit immunoglobulin and goat anti-mouse immunoglobulin antisera were prepared as described in Campbell et al. (1970); monospecific antibodies were obtained by immunosorbent chromatography of these sera on appropriate resin-linked immunoglobulin fractions. Goat anti-rat immunoglobulin antiserum was obtained from Pel-Freez (Rogers, AR). Goat Anti-rabbit IgG-alkaline phosphatase conjugate was obtained from Sigma (St. Louis, MO). Alkaline phosphatase was assayed according to Blake et al. (1984), using 5-bromo-4-chloro- 3-indolyl phosphate as a substrate.

(4) Materials:

Microtiter plates were obtained from the following sources: Polystyrene flat-bottom plates "Immulon 1" from Dynatech (Chantilly, VA); polyvinyl chloride U-bottom and flat-bottom plates "Microtest III" from Becton Dickinson (Oxnard, CA); cellulose ester bottom plates "Millititer HA" from Millipore (Bedford, MA). Detergents and drugs were products of Sigma (St.Louis, MO). Polyethyleneimine was purchased from Eastman Kodak (Rochester, NY).

RESULTS AND DISCUSSION

(1) The Use of Autoradiography and Multiwell Plates in Toxin/Inhibitor Competition Studies - Some Ergonomic Considerations.

The deduction of an inhibition constant for a given ligand by conventional techniques such as the very simple, sensitive, and speedy DEAE-cellulose disk assay requires a large number of manipulations. For a single data point a minimum of 5 pipettings, 5 disk handling steps, 2 counting vial handling steps, and 3 incubation tube handling steps

are required, which for a complete binding curve (consisting of, say, a dozen data points), adds up to approximately 200 individual operations.

The introduction of multiwell titer plates and multichannel pipets permits a vast reduction in the number of sample handling operations. Thus when eight ligands are investigated simultaneously, the number of pipetting steps (for every inhibition constant determined) is reduced by an order of magnitude. A further saving of time and labor can be realized if autoradiographic evaluation replaces single sample counting in the gamma spectrometer. The basic feasibility of this method was demonstrated during the first contract year.

Nevertheless this combination of hybridoma technology and autoradiography does not appear ideal because of the need for a final transfer of the sample out of the multititer plate and onto a sorptive membrane, whose handling (clamping into blotting device; removal therefrom; repeated washes; drying; and autoradiography) requires considerable care and labor. In addition there are drawbacks specific for the membrane chosen (fragility of DEAE-cellulose paper; cost of Nylon derivatives). Therefore, and because of the explicitly stated goals 2b and c of the contract, much of the effort in the second year was devoted to the development of a solid-phase binding assay.

(2) Solid-phase Assay and Autoradiography

(a) Preliminary binding experiments:

Choice of detergent: A prerequisite for a solid-phase is the immobilization of the acetylcholine receptor onto the wall of the container, i.e. the individual wells of a microtiter plate. Undissolved membrane preparations were found to bind to multiwell titer plates rather erratically. We therefore tested detergent-solubilized preparations. Many detergents interfere with protein binding; in fact, Triton X-100 and Tween are frequently used in quench solutions with which to treat surfaces to prevent the nonspecific sticking of proteins. We compared several detergents; results of such an experiment are shown in Fig. 1. Clearly, only cholate-solubilized receptor binds significantly to the PVC plates employed. This is somewhat surprising as deoxycholate, which differs from cholate only by a hydroxyl function, completely blocks receptor adsorption (it could also be argued that most detergents interfere not with receptor-plastic interaction, but with the subsequent binding of ^{125}I-alpha-bungarotoxin to the receptor; however, many of the detergents tested, such as Triton X-100 and deoxycholate, have been in use for years for the extraction of active acetylcholine receptor from electric tissue and muscle). For all subsequent

experiments, Torpedo receptor was used extracted from electric organ membranes by means of 1% cholate.

Choice of titer plate: Not all multiwell titer plates are equally suited for autoradiographic evaluation. Three plate types were tested and found to differ greatly in their receptor binding activity (Fig.2). PVC plates exhibit better receptor-sorptive properties than the commonly used polystyrene plates and are easily cut with scissors. This latter feature permits quick removal of the rim of the plate so that the bottoms of the wells can be brought in direct contact with film for autoradiography; in addition, autoradiographic results can be readily corroborated by counting individually cut wells in a gamma spectrometer. Millipore's Millititer plates exhibit by far the highest protein sorptive capacity, but suffer from two major drawbacks. One of these is an ergonomic one: direct analysis, either by autoradiography or ELISA, is not possible, and radioactivity measurements require the laborious punching out of individual well bottoms. The other is economic: cost for a single plate exceeds $ 8.- Therefore most studies were performed using PVC plates, initially in the U-bottom version; later on flat-bottom plates were used almost exclusively.

(b) Optimization of the autoradiographic assay

Optimal receptor concentration: The protein-sorptive capacity of a PVC well is limited to approximately 1 microgram. When increasing quantities of Torpedo membrane extract are applied, bound receptor first increases, then levels off and eventually even falls, presumably because the receptor (which, after all, represents only a relatively small fraction of the total protein) is competed off by more strongly binding proteins (Fig.3). Optimal adsorption was seen with diluted extracts containing approximately 0.5 picomole of toxin binding site.

Enhancement of receptor adsorption: Since only a rather small fraction (ca 7%) of applied receptor binds to PVC wells even under the optimal conditions described above, several nonspecific treatments of the plates prior to the receptor adsorption step were explored. Polyethyleneimine has frequently been employed for the treatment of filters in receptor ligand assays as it reduces nonspecific ligand binding without compromising receptor binding. We tested several concentrations of this polycation and found that it increased receptor binding by 3 to 4fold (Fig.4). Polyethyleneimine has the added advantage that nonspecific toxin binding to PVC is sharply reduced even if plates are not quenched (with protein solutions such as 3% casein) prior to receptor addition. Another polycation, 6,6-ionene, a polymer made up of hexamethonium moieties and carrying permanent positive charges in each 7th position along the

chain, failed to give increased signals, possibly because it can directly block the toxin binding site on the receptor. Concanavaline A was also investigated as a potential anchoring agent; it did not noticeably improve receptor binding, most probably because too many Torpedo membrane proteins, being reactive glycoproteins themselves, compete for this lectin (data not shown).

(c) Improvement of the autoradiographic procedure

Initially plates with pointed wells were used which give rise to fairly diffuse signals (see e.g. Fig.3). To render subsequent quantitative analysis (by means of densitometry) simpler, we switched to flatt-bottom plates. Since standard exposure casettes cannot hold objects as thick (>8 mm) as the titer plates, we now use a custom-built casette constructed from black plexiglass and lined with black photographer's felt; it holds a 20.3 x 25.4 cm film for the simultaneous exposure of 4 plates. Larger boxes to hold 35.6 x 43.2 cm films for the simultaneous processing of 10 to 15 plates could easily be built. A special problem arose due to the optical properties of the plates: even in dim darkroom light they will produce images unless covered. We now routinely cover the plates with aluminum foil. Preflashing is not required. To reduce the autoradiographic halo around each well the volumes of receptor-containing extract are kept to a minimum (25 ul or less); further reduction may be accomplished with a metal shielding device. Results of an 8-ligand experiment are shown in Fig.5. It is obvious from this experiment that ligands capable of binding to the toxin site on the receptor are readily recognizable, and that furthermore their affinity for the receptor can be estimated directly from the autoradiograph. The inhibition constants deduced are higher than would be expected from their known affinities. This is due to the irreversible association of the toxin with the receptor which precludes any true equilibrium measurement (at equilibrium all receptor sites would be occupied by toxin); as a consequence, protection constants are measured (= concentrations of drug that reduce the initial rate of toxin binding to half), which tend to be higher than true binding constants, especially when prolonged incubations have to be carried out to get sufficient toxin binding (Weber and Changeux, 1974). Film saturation at high sample radioactivities and/or excessive exposure times could cause problems; however even the rather strong signals of Fig.5 were shown to fall in the linear range of film response. As shown in Fig 5b, results of direct ^{125}I measurements in a gamma spectrometer and densitometric data agree very well.

At this point the autoradiographic version of the multiwell titer assay is ready for further automation which we expect to accomplish by means of a pipetting station and computer analysis of densitometric data.

(3) The Use of ELISA and Multiwell Titer Plate Technology in Receptor-Ligand Interactions.

As a rule colorimetric assays are far less sensitive than assays involving radiolabeled compounds. In our specific case this also holds. Whereas as little as 1 femtomole of radioiodinated toxin is readily detectable, about a hundred times more immunoglobulin is needed for detection by an alkaline phosphatase-coupled secondary antibody. We have worked on two versions of an ELISA assay for the detection of receptor-bound bungarotoxin (see below) both of which require efficient adsorption of receptor onto titer plate wells.

(a) Experiments on increased receptor adsorption

Nonspecific anchoring methods (e.g. coating with polyethyleneimine) increase adsorbed receptor levels toward the threshold of detectability by ELISA methods, but not significantly beyond it. A further increase in receptor density on the well wall cannot be achieved without either purifying the receptor itself or an anchoring agent specific for it. Receptor purification does not appeal because of the limited supply of electric tissue, and the technical difficulty and low yield of the purification procedures. The same reasons, a fortiori hold for the use of monospecific polyclonal anti-receptor antibodies (whose purification requires the purification of the receptor for immunization as well as affinity resin synthesis); this is regrettable since nonospecific anti-receptor antibodies raise receptor adsorption to beyond the picomole/well mark (see Fi.6). A receptor-specific monoclonal antibody which can be easily prepared and purified in the laboratory appears as a workable compromise.

(b) Preparation and isolation of mAb35

The choice of mAb35 is dictated by several considerations: The hybridoma secreting it, originally developed in the laboratory of Lindstrom, is available from ATCC (under the designation TIB175); the antibody, although originally raised against the receptor from Electrophorus electricus, has high affinity for the Torpedo receptor; and finally, we have had some experience with it using it in our work on chick muscle receptor (Ross et al., 1987). We found that the hybridoma grows much better in PRMI than in DME as suggested by the suppliers. Since the antibody constitutes only a small fraction of the total protein in the culture supernatant, the supernates themselves are unsuitable for the coating of titer plates. The monoclonal antibody must be purified or at least considerably enriched. Several purification protocols were explored.

Conventional protein fractionation procedures: Following the suggestions of Lindstrom we tested a combination of conventional fractionation procedures for the isolation of mAb35. Supernates were first concentrated by ultrafiltration and ammonium sulfate precipitation, and the immunoglobulin fraction applied to DEAE-cellulose and eluted with a linear salt gradient. Little useful fractionatioen was accomplished. Affinity chromatography: mAb35 interacts strongly and specifically with the acetylcholine receptor and, since it is a rat immunoglobulin, with anti rat IgG antibodies as well. Both procedures have been used. For reasons of economy the latter technique is preferable. As shown in Fig.7, the antibody significantly enhances receptor binding to PVC wells.

(c) Detection of unblocked binding sites

Anti-toxin antibodies: In the autoradiographic version of the solid phase assay, radiolabeled toxin indicates the presence of an unliganded acetylcholine binding site. If unlabeled toxin is used in the assay, it can be detected with anti-toxin antibodies after treatment with secondary antibody conjugated to a reporter enzyme. Preliminary results of this approach are shown in Fig.8. Signals are faint, but results clearly indicate that the color reaction depends on the presence of receptor, toxin, and anti-toxin and on the absence of inhibiting ligands. Obviously more work is required for the optimization of the procedure. A major problem with this approach is the large number of the sequential incubations and washes required (polyethyleneimine / receptor / quench solution / ligand and toxin / anti-toxin / secondary antibody-toxin conjugate / enzyme substrate). Although all of these steps, including colorimetric analysis, could be automated, the number of manipulations appears excessive.

Toxin-enzyme conjugates: A simplification of the above protocol involves competition of ligand and a toxin-enzyme conjugate. Such conjugates were prepared with alpha-bungarotoxin as well as the alpha toxin from Naja naja siamensis venom, but lacked high-affinity receptor binding, possibly because of excessive covalent modification of amino functions in the toxin moiety by the glutaraldehyde used as a linking agent.

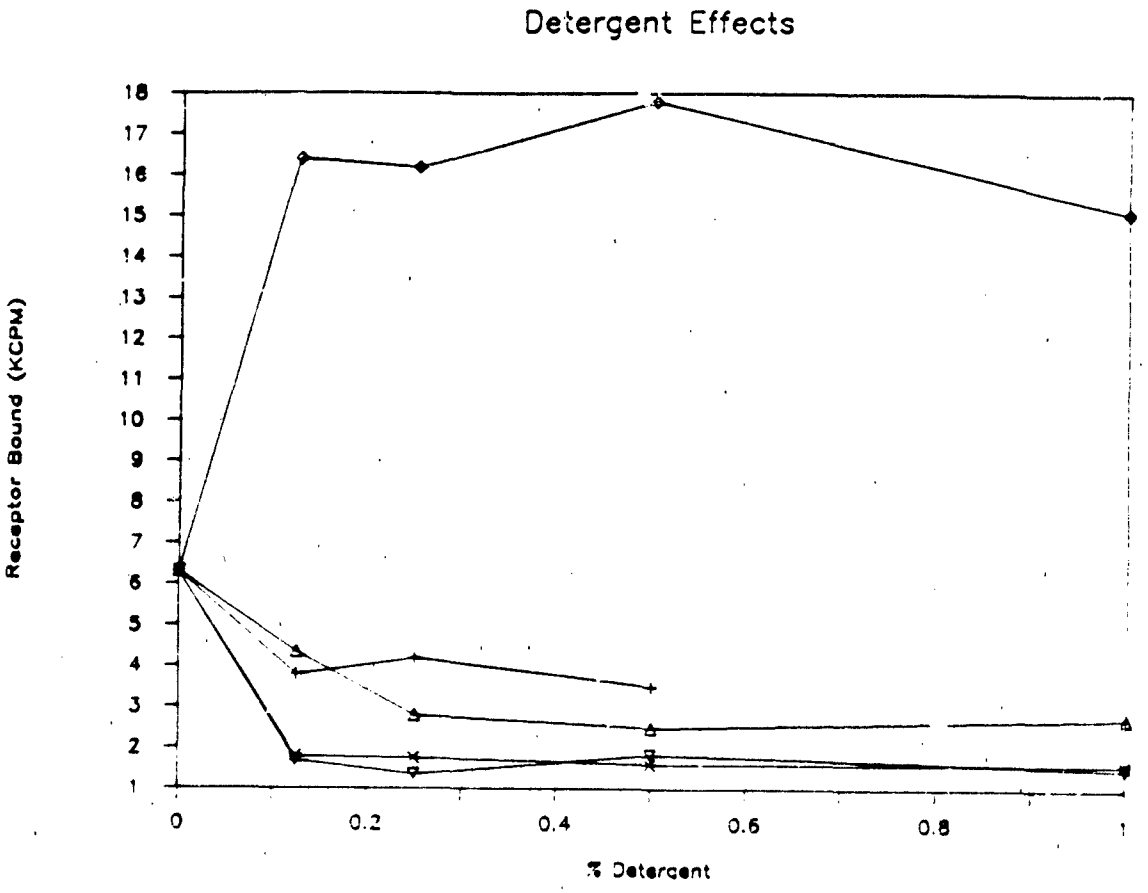


Fig. 1 Binding of Acetylcholine Receptor as a Function of Detergent Type and Concentration

Torpedo membranes were prepared as described in the Methods section and diluted 1:500 into PBS containing detergent (CHAPS: ▽; sodium cholate: ◇; sodium deoxycholate: + ; digitonin: X ; and Triton X-100: ⤳) at the indicated concentrations (v/v). Aliquots of 100 ul were pipetted into individual wells of a PVC microtiter plate and left standing for 1 h at room temperature. Wells were then quenched with 3% casein for 10 min and incubated with ^{125}I-alpha-bungarotoxin (10^{-8} M) for an additional h. After three washes, individual wells were cut off the plate and counted in a gamma spectrometer.

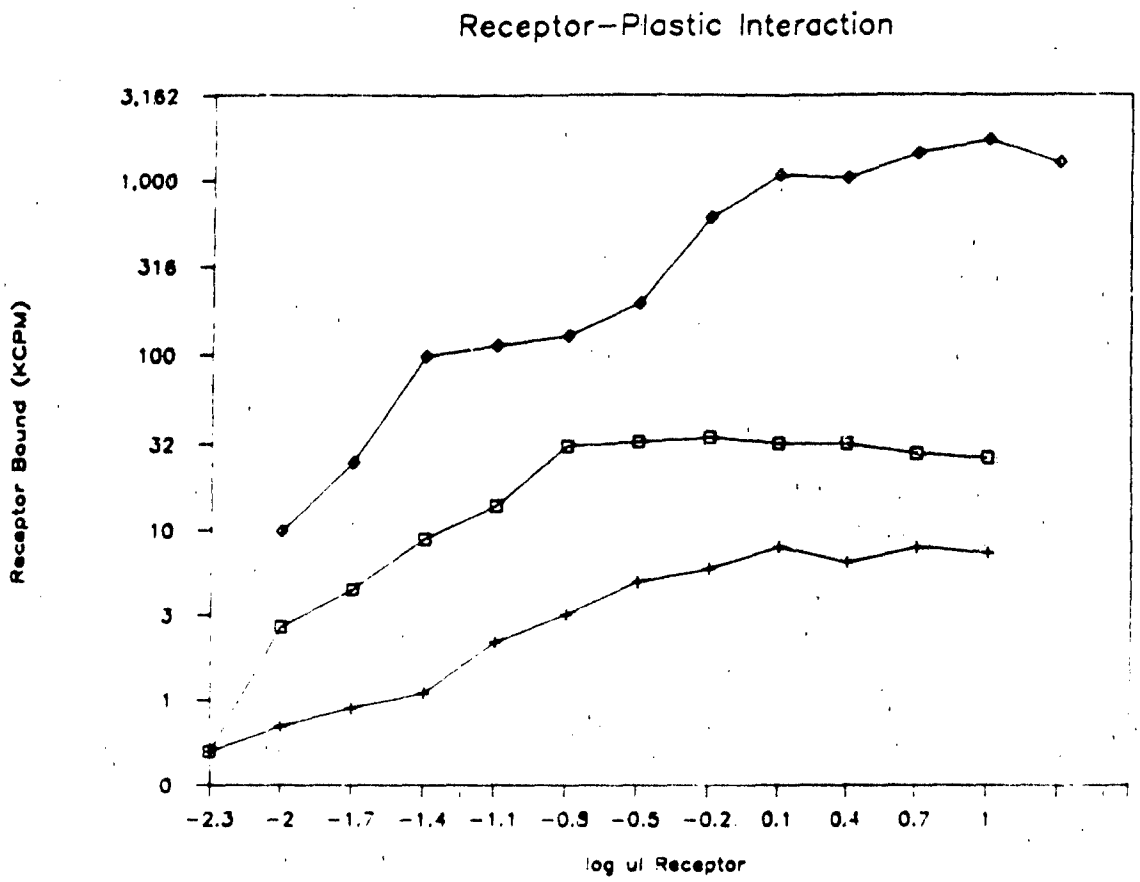
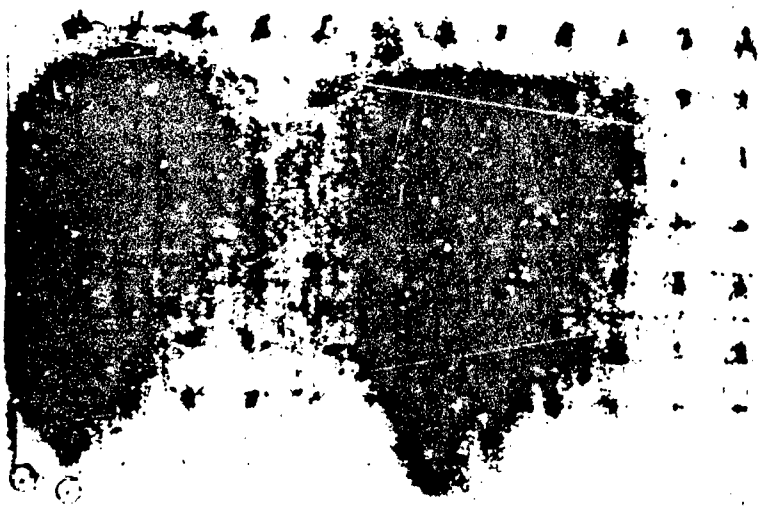


Fig. 2 A Comparison of Different Multiwell Titer Plates

Torpedo membranes were dissolved in 9 volumes of cholate buffer and 100 ul of this concentrated sample and of serial (1:2) dilutions therof pipetted into wells of plates made of polystyrene (+, Dynatech Immulon 1), flexible polyvinyl chloride (□, Falcon Microtest III), and polystyrene with mixed cellulose ester membrane bottoms (◇, Millipore, Millititer HA). After quenching with 3% casein, wells were incubated with 5×10^{-9} M ^{125}I-alpha-bungarotoxin for one h, washed, and bound radioactivity determined by: washing wells twice with 0.2 ml 1% SDS/1 N NaOH, transferring the washings to a tube and counting (polystyrene); cutting off and counting individual wells (PVC); or punching out the membrane and counting (Millititer).



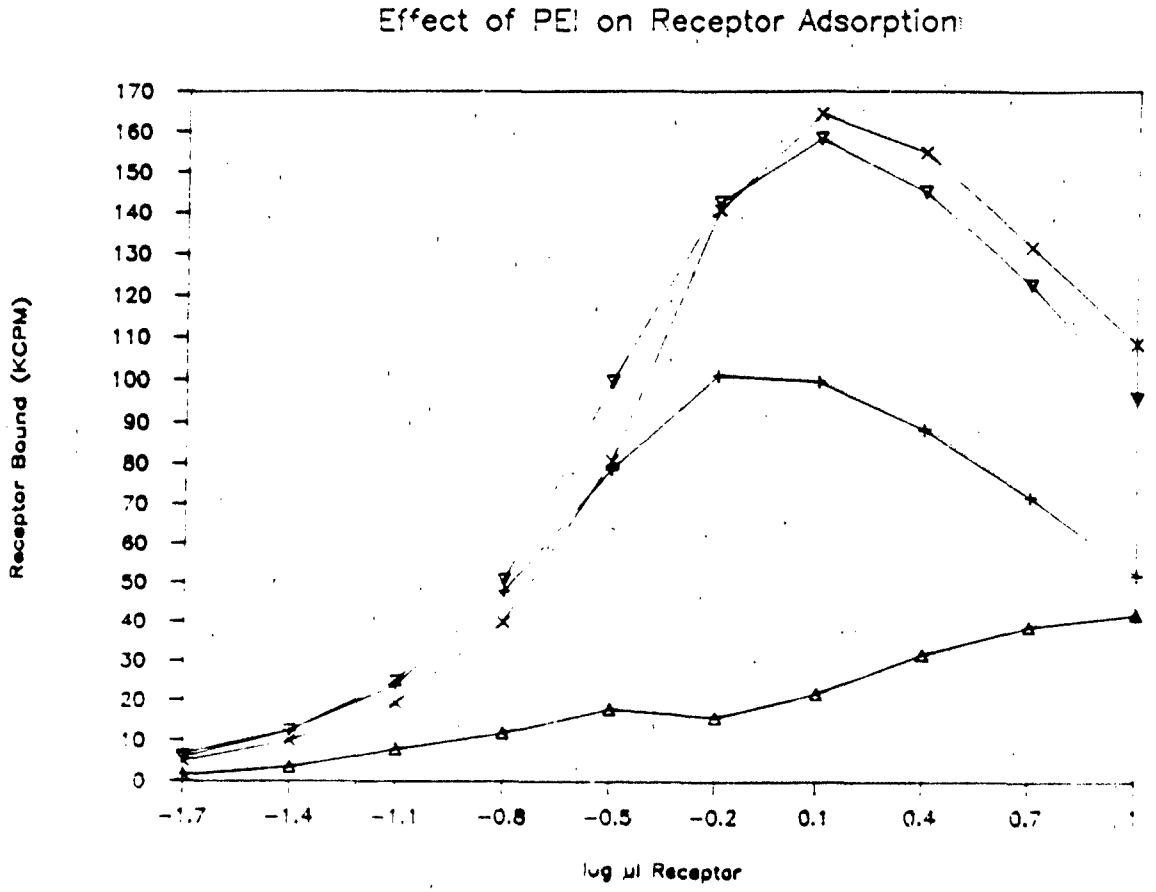


Fig. 4 Effect of Polyethyleneimine on Receptor Adsorption

Wells of a PVC plate were treated with PBS (▲); and 0.005% (+); 0.05% (▽); and 0.5% polyethyleneimine (×). After a brief wash with PBS, Torpedo receptor, serially diluted into cholate buffer, was added as described in the legend to Fig. 2. Overnight incubation was followed by quench with 3% casein, incubation with ^{125}I-alpha-bungarotoxin, wash and determination of bound radioactivity.

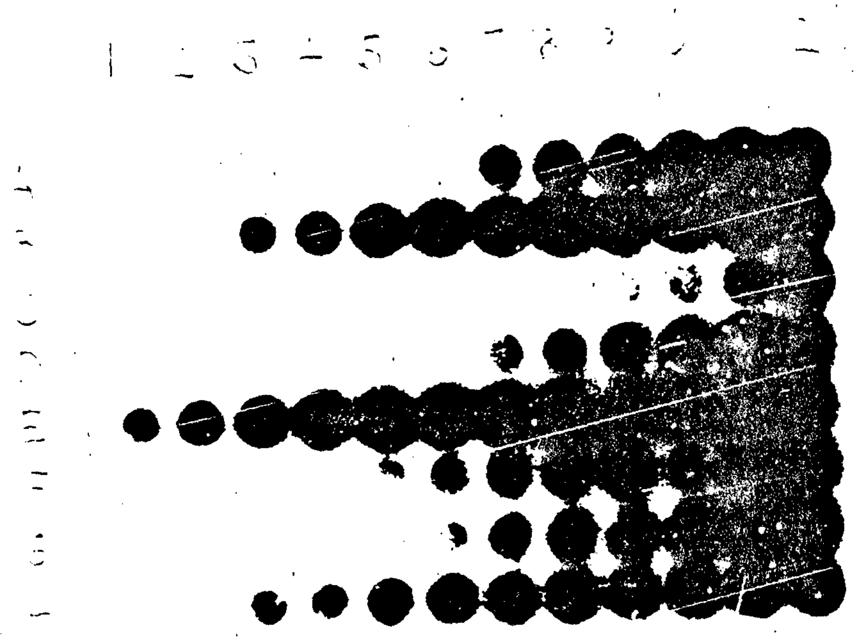


Fig. 5 Ligand Inhibition Measured by a Solid-Phase Autoradiographic Assay.

A [illegible] briefly treated with 0.05% [illegible] coated with [illegible] receptor by overnight [illegible] incubation [illegible] (1:3.16) diluted [illegible] into [illegible] the highest concentration, and well [illegible] lowest, with well [illegible] serving as ligand-free control. Ligands tested were: A, [illegible] acylcholine (1 [illegible]; B, carbamoylcholine (initial concentration: 1 [illegible]); C, [illegible] alpha toxin (0.1 [illegible]); D, cytisine (0.[illegible]); E, sodium chloride (1 M); F, nicotine (1 M); G, d-tubocurarine (0.1 M); H, decamethonium [illegible]. After addition of [illegible] toxin to a final concentration of 1.5x10[illegible] M, incubation was continued for another h. Then all wells were washed repeatedly with PBS, and the plate subjected to autoradiography as described.

densitometry and spectrometry compared

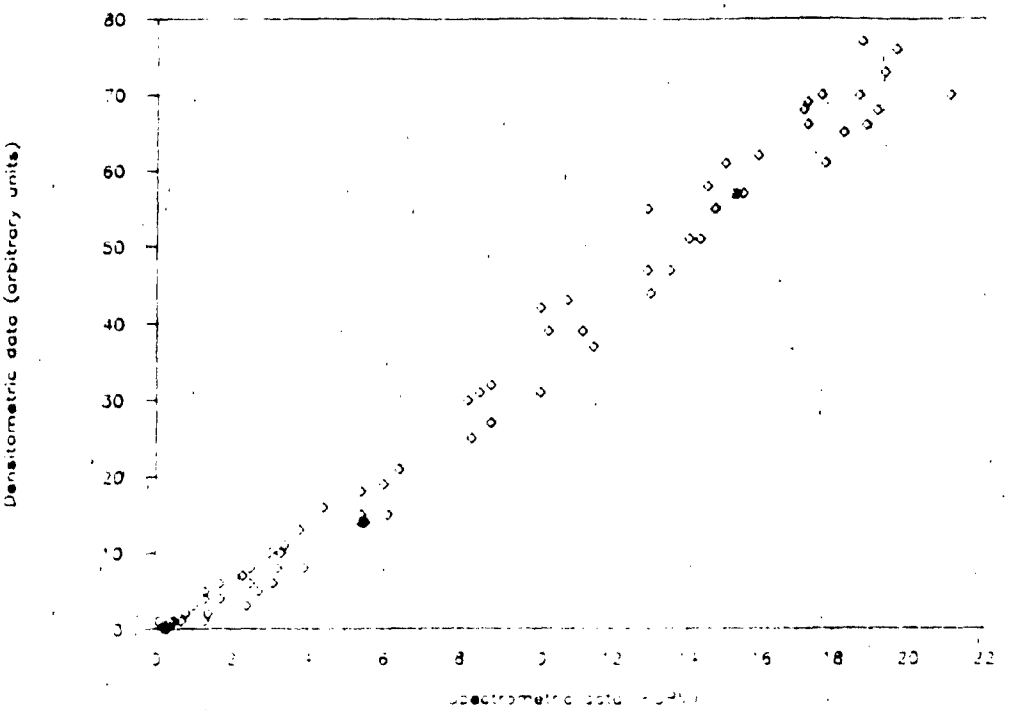


Fig. 8b [illegible] densitometric and spectrometric [illegible]

The microdensitograph described in Fig.5 was used to densitometrically scan the automatic linear scanning mode of the Joyce-Loebl Chromoscan 3 densitometer. The FUC plate was then individual cells and counted in an LKB 1282 Compugamma spectrometer. Optical densities in arbitrary units are plotted against [illegible] counts. The correlation coefficient was determined to be 0.95.

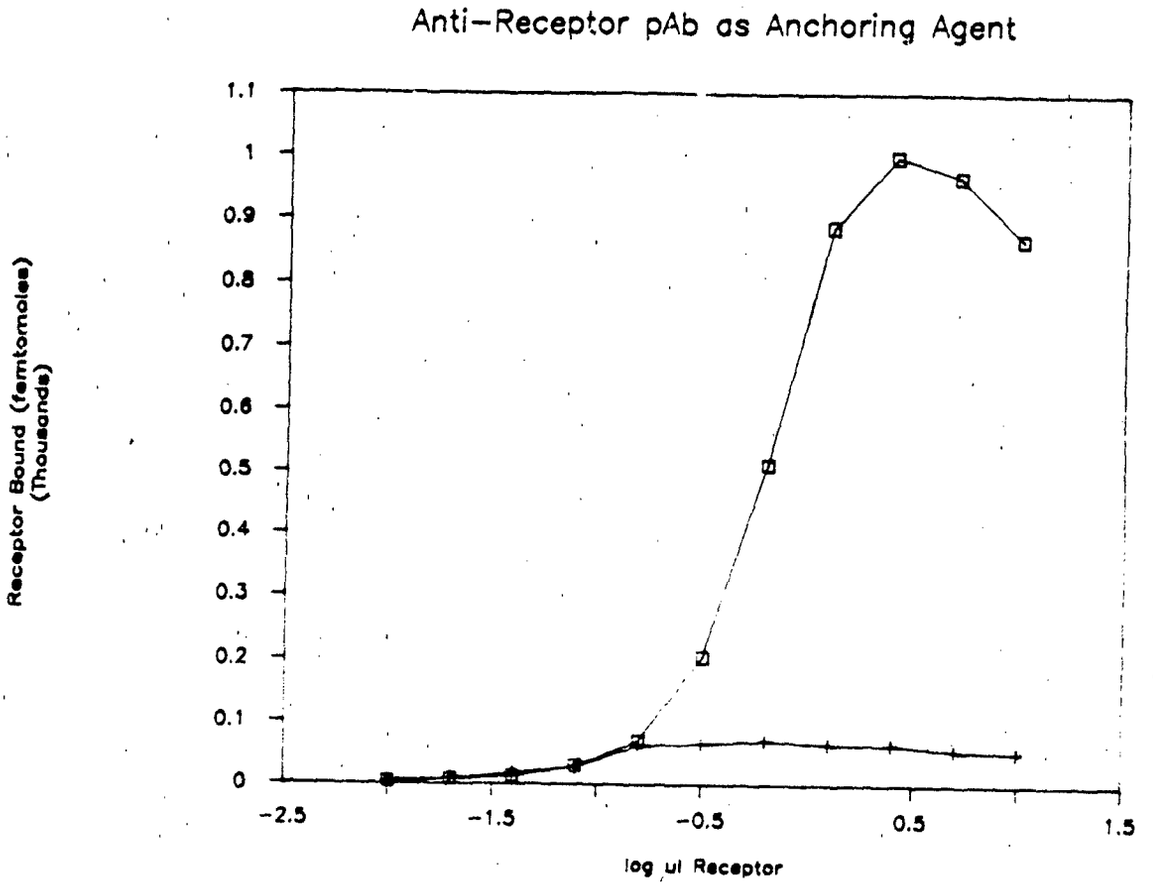


Fig. 6 Monospecific Polyclonal Anti-Receptor Antibodies as Anchoring Agents

PVC titer plate wells were treated, at 4° overnight, with 100-ul aliquots of a 10^{-7} preparation of monospecific anti-receptor antibodies (10^{-7} M). After washing, wells were incubated with serial dilutions of *Torpedo* membrane extract, followed by incubation with ^{125}I-alpha-bungarotoxin (10^{-7} M). Values are corrected for background binding observed in the presence of 1 mM d-tubocurarine. Binding of receptor in the absence of antibody (+, from Fig.2) is given for comparison.

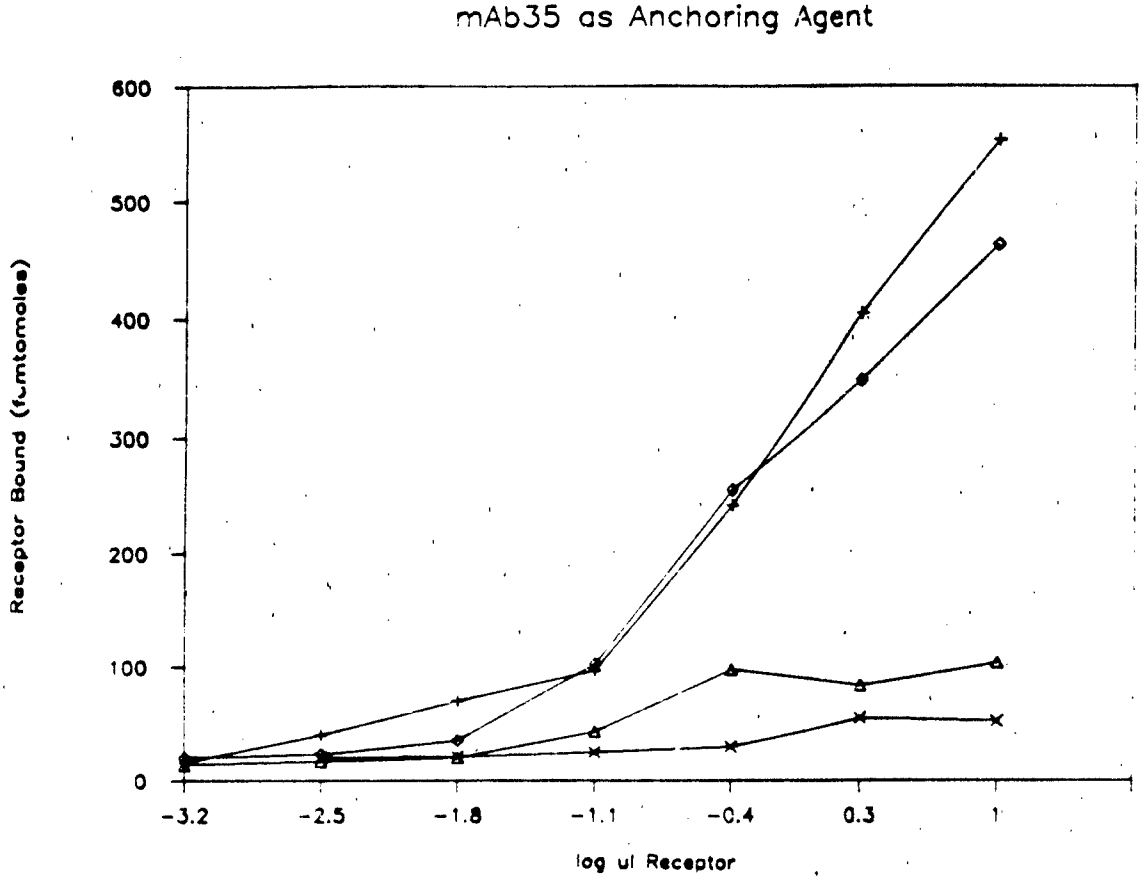


Fig. 7 Receptor Binding to mAb35-Coated Wells

PVC titer plate wells were treated, at 4° overnight, with 100 ul of ca. 5×10^{-7} M (+); 10^{-7} M (◇); 2×10^{-8} M (△); and 4×10^{-9} M (×) mAb35. After washing, wells were incubated with serial (1:5) dilutions of Torpedo membrane cholate extract (highest concentration: 10^{-7} M) for another 24 h at 4°. Then wells were quenched with 3% casein and incubated with ^{125}I-alpha-bungarotoxin (4×10^{-7} M). After an additional hour, wells were washed, cut and counted.

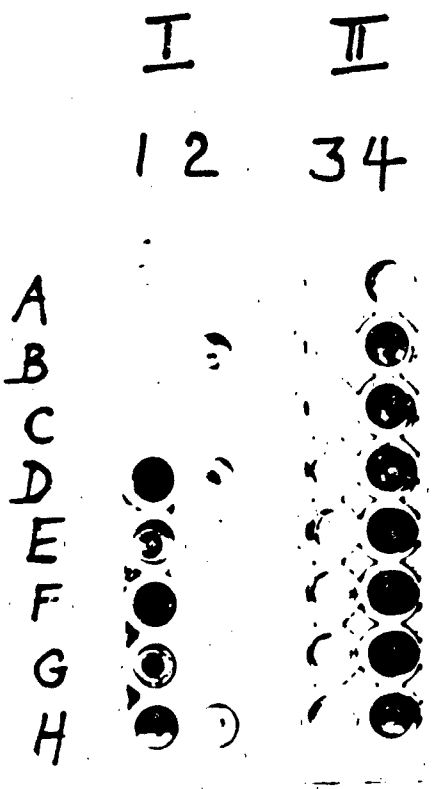


Fig. 8 Preliminary ELISA Results

Strip I: All wells of a polystyrene plate were coated with mAb35 without (A,C,E,G) or with (B,D,F,H) polyethyleneimine pretreatment. Wells were then incubated with 0 (A,B); 0.1 (C,D); 1.0 (E,F); and 10 (G,H) picomoles of Torpedo receptor and quenched with 3% casein. Then 4 picomoles of ^{125}I-alpha-bungarotoxin were added without (column 1) or with (column 2) 1 mM d-tubocurarine. After 30 min at room temperature the contents were removed and 1 ug monospecific anti-toxin was added for a 2-h incubation followed by a 1-h treatment with the secondary antibody-alkaline phosphatase conjugate. Enzyme was visualized as described in the methods section, and the plate photocopied.

Strip II: All wells were treated with 0.05% polyethyleneimine; mAb35; and receptor (0.5 picomole). Then d-tubocurarine was added to row A at 100 mM and serially (1:5) diluted to rows B, C $_{-8}$ etc., foolowed by ^{125}I-toxin (final concentration 1.5×10^{-8} M). After 30 min contents were removed and further treatments resembled those described for strip I, except that the anti-toxin treatment was left out in column 3.

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