ANALYTICAL BIOCHEMISTRY 130, 1–8 (1983)

Analysis of Receptor–Ligand Interactions Using Nitrocellulose Gel Transfer: Application to *Torpedo* Acetylcholine Receptor and Alpha-Bungarotoxin

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Received April 12, 1982

A nitrocellulose–gel transfer technique has been adapted to study the interaction of a polypeptide ligand with individual receptor subunits. The acetylcholine receptor isolated from *Torpedo californica* has been separated into its subunits by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred in a renaturing environment to nitrocellulose sheets. The sheets were incubated with ^{125}I-alpha-bungarotoxin and autoradiographed. A single receptor polypeptide, the alpha subunit (40K) bound the labeled toxin. This binding was demonstrated to be both saturable and specific, although the affinity of ^{125}I-alpha-bungarotoxin (K_D, 165 nM) and the potency of *d*-tubocurarine to displace this binding (IC_{50}, 1 mM) were both reduced by several orders of magnitude when compared to the native receptor.

Peptide and polypeptide neuromodulators and hormones play an important role in chemical communication between cells. In order to exert their biological effects, these mediators must first bind to specific receptor proteins on the plasma membrane resulting in the activation of an appropriate physiological signal. An important key to understanding the mechanisms involved requires the identification of the polypeptide in which the recognition site resides. Since receptors are often present in low concentrations in a complex mixture of other proteins, identification of these receptors presents an important challenge.

A highly effective method for separating such complex mixtures of membrane polypeptides is SDS[1]–polyacrylamide gel electrophoresis. However, during this process the polypeptides are denatured and presumably lose their ligand-binding properties. To circumvent these effects of SDS, affinity probes have been used to label covalently the receptor polypeptide(s) prior to electrophoresis. However, this technique has a number of inherent disadvantages, the major ones being the difficulties in designing and synthesizing a ligand probe with the specificity and reactivity sufficient to covalently attach only to the appropriate receptor polypeptide (1).

Bowens *et al.* (2) have successfully demonstrated that nuclear histone polypeptides resolved by SDS–gel electrophoresis and transferred to nitrocellulose sheets in a renaturing environment bound ^{32}P-labeled DNA. We have examined whether this technique could be adapted to study the interaction of transferred receptor proteins with a polypeptide ligand. We chose alpha-bungarotoxin, a polypeptide isolated from the venom of *Bungarus multicinctus,* since it binds with high affinity and specificity to the nicotinic acetylcholine receptor from *Torpedo californica,* and this interaction has been extensively characterized (3–7). The results presented here demonstrate that following SDS–gel electrophoresis and transfer of the resolved proteins

[1] Abbreviations used: SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; VSV, vesicular stomatitis virus.

0003-2697/83 $3.00

to nitrocellulose sheets, a single polypeptide having the molecular weight of the alpha subunit bound ^{125}I-alpha-bungarotoxin in a saturable and specific manner.

MATERIALS AND METHODS

Receptor and ligand. Acetylcholine receptor-enriched membrane fragments from *T. californica* were prepared according to the method of Sobel *et al.* (8). This preparation bound 5.6 nmol of ^{125}I-alpha-bungarotoxin/mg of protein as determined by a Millipore filtration assay (3). For some of the initial experiments these membrane fragments were further purified by affinity chromatography. Protein concentrations were determined according to a modification of the Lowry method (9). ^{125}I-Alpha-bungarotoxin, sp act 93 Ci/mmol, was purchased from New England Nuclear Corporation.

SDS–gel electrophoresis and protein transfer. SDS–polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (10). Acrylamide (Bio-Rad Laboratories) was purified by treatment with Amberlite MB-1 (Sigma Chemical Co.). The separating gel (0.8 mm thick) contained 4 M urea, 0.1% SDS, 12% acrylamide, pH 8.8 (Tris). The stacking gel contained 4 M urea, 0.1% SDS, 5% acrylamide, pH 6.8 (Tris).

Both radiolabeled and unlabeled molecular-weight markers were used. ^{35}S-Labeled coat proteins of vesicular stomatitis virus (VSV) were synthesized in a rabbit reticulocyte cell-free system from the viral RNA, and the molecular weights of the major radioactive bands were 43K, 45K, 50K, and 61K (11). Bovine serum albumin, (68K) and ovalbumin (43K) were the unlabeled molecular-weight markers. Acetylcholine receptor samples and molecular-weight markers were treated with sample buffer (10% glycerol, 2.0% sodium dodecyl sulfate, and 0.0625 M Tris, pH 6.8) at room temperature for 30 min prior to electrophoresis.

Following electrophoresis, SDS was removed from the gel by immersion for 3 h in a urea-containing buffer (20 mM NaCl, 2 mM Na_2–EDTA, 4 M urea, and 10 mM Tris, pH 7.0). The gel was placed between two nitrocellulose sheets (Schleicher and Schuell, BA85) and the proteins were transferred by diffusion in a urea-free buffer using the procedure of Bowens *et al.* (2) with the following modifications. The nitrocellulose "gel sandwich" was placed in transfer buffer (prepared without DTT) for 16 h at which time fresh buffer was added for an additional 44 h. The extent of transfer of proteins was examined by staining the gel with Coomassie blue, and one of the nitrocellulose sheets in 0.1% aniline blue–black, and also by autoradiography of the transferred ^{35}S-labeled VSV coat proteins.

^{125}I-Alpha-bungarotoxin binding to polypeptides adsorbed onto nitrocellulose sheets. Following transfer of the resolved polypeptides, the nitrocellulose sheet (12 × 12 cm) was preincubated for 9 h in 100 ml of prebinding buffer (20 mM NaCl, 1 mM Na_2–EDTA, 10 mM Tris, pH 7.0, and 4.0% BSA). This step substantially reduces the amount of labeled toxin that adsorbs to the nitrocellulose sheets. The sheet was then placed in a plastic pouch (Seal-a-Meal), and 5.5 ml of 10 nM ^{125}I-alpha-bungarotoxin in 1 mM Na_2–EDTA, 10 mM Tris, pH 7.0, 1.0% BSA, and 20 mM NaCl was added. The sealed pouches were incubated for 4 h with agitation. Following this incubation period the sheets were washed 10 times, 1 min each, with ice-cold wash buffer (1 mM Na_2–EDTA, 10 mM Tris, pH 7.0, 200 mM NaCl, and 0.2% BSA). This wash procedure has been optimized to remove selectively ^{125}I-alpha-bungarotoxin that is rapidly dissociable.

In order to examine in detail ^{125}I-alpha-bungarotoxin binding, the above method was modified in the following ways. A large single well, 8 cm wide and capable of holding 600 μl of sample, was formed in the stacking gel. Following electrophoresis and transfer of proteins as described above, the nitrocellulose sheets, each containing equivalent amounts of the adsorbed polypeptides, were sectioned into 1 × 12-cm strips. End strips were dis-

carded. Each strip was then placed into a separate Plexiglas incubation chamber (1.3 cm wide × 13.5 cm long, 2 cm high) and preincubated in 5 ml of 4.0% BSA overnight. The strips were then incubated for 30 min with varying concentrations of cholinergic ligands. At this point ^{125}I-alpha-bungarotoxin was added to a final concentration of 10 nM and the incubation continued for an additional 4 h.

The position of the bound ^{125}I-alpha-bungarotoxin was determined following extensive washing of the strips and subsequent autoradiography as described for the whole nitrocellulose sheet. The amount of ^{125}I-alpha-bungarotoxin bound was determined by excising the radioactive band and measuring gamma emission. In order to estimate the amount of ^{125}I-alpha-bungarotoxin that adheres to the nitrocellulose strip, an equivalent area of the nitrocellulose strip lacking the radioactive band was also counted. This value was subtracted from the total radioactivity to give the amount of labeled toxin bound to the alpha subunit alone.

The technique was further modified to minimize incubation volumes and to obtain high concentrations of alpha-bungarotoxin (up to 500 nM) necessary to achieve saturation. This smaller incubation volume (600 μl) was made possible by incubating only that portion of the nitrocellulose sheet containing the adsorbed alpha subunit. The position of alpha was determined by incubating strips cut from each end of the nitrocellulose sheet with the labeled toxin followed by autoradiography. By aligning the autoradiograph of the end strips with the nitrocellulose sheet, it was thus possible to cut out a horizontal section of the sheet that contained the alpha subunit. This nitrocellulose section was then cut into 1-cm strips and incubated with increasing concentrations of ^{125}I-alpha-bungarotoxin. Following a 4-h incubation and autoradiography, the radioactive band was excised, counted, and the radioactivity in an equivalent area subtracted to give the ^{125}I-alpha-bungarotoxin bound to the alpha subunit. In order to estimate the nonspecific binding component, incubations were also conducted in the presence of a 100-fold excess of the unlabeled alpha-bungarotoxin.

RESULTS

Transfer of Acetylcholine Receptor Polypeptides from SDS–Polyacrylamide Gels to Nitrocellulose Sheets

Affinity-purified nicotinic acetylcholine receptor from *T. californica* was resolved by SDS–gel electrophoresis into four polypeptide subunits of apparent molecular weights of 40,000 (alpha), 50,000 (beta), 60,000 (gamma), and 65,000 (delta) and were transferred to nitrocellulose sheets. Polypeptides were transferred by diffusion under conditions designed to promote renaturation (12,13). One of the duplicate filters was stained with aniline blue–black (Fig. 1A) to locate the position of the transferred polypeptides. The other filter was incubated with ^{125}I-alpha-bungarotoxin (Fig. 1B) to assess if any of the subunits adsorbed on the nitrocellulose bound the radiolabelled ligand. The four subunits and molecular-weight markers are shown in Fig. 1A. The observed pattern faithfully replicates the original gel on which virtually no proteins remained.

Autoradiography of the nitrocellulose sheet (Fig. 1B) obtained following incubation with 10 nM ^{125}I-alpha-bungarotoxin demonstrates that only one of the transferred polypeptides bound the ligand. This polypeptide is the alpha subunit (14) as determined by its relative mobility as compared to the ^{35}S-labeled marker VSV coat proteins (Fig. 1B, lane d). The sensitivity of this binding is illustrated by the observation that amounts of the alpha subunit so small as to be undetected by staining can be easily seen using the radioactive ligand (Figs. 1A and B, lane f). The selectivity of this technique is illustrated by the absence of ligand binding to proteins present in high concentrations (Fig. 1A, lanes a,b, and d). The ^{35}S-labeled viral coat proteins, in addition to

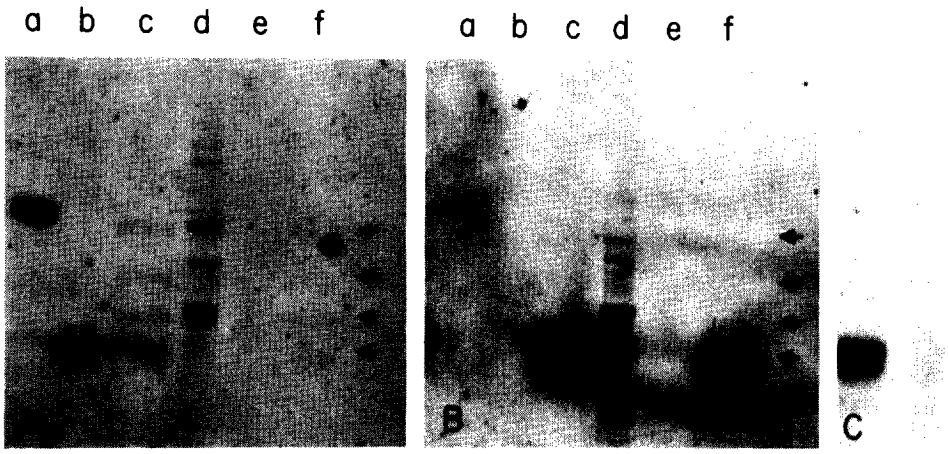

FIG. 1. Transfer of the affinity-purified *T. californica* acetylcholine receptor polypeptides to duplicate nitrocellulose sheets and binding of ^{125}I-alpha-bungarotoxin to the alpha subunit. (A) Nitrocellulose sheet stained with 0.1% aniline blue–black following transfer of polypeptides from a polyacrylamide gel. Lanes a and b contain unlabeled molecular-weight markers, BSA (3.9 μg) and ovalbumin (10 μg), M_r 68,000 and 43,000, respectively. Lane c and f contain 7.2 and 2.4 μg, respectively, of *T. californica* receptor separated into polypeptides of apparent M_r (from bottom to top) 40,000 (alpha), 50,000 (beta), 60,000 (gamma), and 65,000 (delta); lane d contains polypeptides from the reticulotye lysate used in the cell-free translation of VSV-mRNA; lane e is blank, The arrows denote (from bottom to top) the positions of the alpha, beta, gamma, and delta subunits). (B) Autoradiograph (3-day exposure) of the duplicate nitrocellulose sheet following a 4-h incubation with 10 nM ^{125}I-alpha-bungarotoxin. Lanes c and f show the binding of ^{125}I-alpha-bungarotoxin to a single polypeptide whose position corresponds to the alpha subunit in corresponding lanes c and f, Fig. 1A. The intensity of the bands reflects the amount of receptor proteins added to the polyacrylamide gel. Lane d, the ^{35}S-labeled viral coat proteins used as molecular-weight markers. The lowest of the major bands is the N protein (molecular weight, 43K). (C) Autoradiograph (40-h exposure) of two individual nitrocellulose strips (each containing 5 μg of protein of *T. californica* acetylcholine receptor) which were excised from a whole sheet. The strip on the right was preincubated with unlabeled 1 μM alpha-bungarotoxin. Both strips were then incubated with 10 nM ^{125}I-alpha-bungarotoxin.

serving as molecular-weight markers, illustrate the fidelity of protein transfer and allow the evaluation of the equality of transfer to each nitrocellulose sheet.

In order to examine whether excess unlabeled alpha-bungarotoxin could displace ^{125}I-alpha-bungarotoxin, strips containing equal amounts of the alpha subunit were incubated in the presence and absence of 1 μM alpha-bungarotoxin. In the presence of cold toxin, virtually no label could be observed in the autoradiograph (Fig. 1C, right). This indicates that the binding of ^{125}I-alpha-bungarotoxin is not simply a nonspecific adsorption, but is due to an interaction of the radiolabeled ligand with a limited number of sites on the alpha subunit.

Characterization of the Interaction of ^{125}I-Alpha-Bungarotoxin with the Nitrocellulose-Bound Alpha Subunit

In order to examine the specific component of ^{125}I-alpha-bungarotoxin binding in detail, sections of the nitrocellulose sheets containing equal amounts of the alpha subunit were excised. They were then incubated with increasing concentrations of the labeled toxin (10–600 nM). These sections were then counted by gamma emission and the radioactivity of an equivalent area lacking the alpha subunit was subtracted to give an estimate of the binding to the alpha subunit alone. The total binding to the alpha subunit was plotted as a function of the concentration of

the labeled toxin in the incubation mixture. In order to determine the specific component of binding, labeled toxin binding was also measured in the presence of 100-fold excess of the nonradioactive alpha-bungarotoxin. Figure 2 illustrates that over the entire concentration range examined, the specific component constitutes over 90% of the total binding observed. The dissociation constant (K_D) of this specific component was estimated from the concentration of ^{125}I-alpha-bungarotoxin which gave half the maximal binding and was about 165 nM. This value is considerably greater than the K_D observed for the intact receptor (3) and possible reasons for this discrepancy are presented under Discussion.

Further evidence of specificity was demonstrated by examining the ability of cholinergic ligands to displace the binding of the labeled toxin from the nitrocellulose-bound alpha subunit. Unlabeled alpha-bungarotoxin, *d*-tubocurarine (a competitive nicotinic antagonist), and atropine (a competitive muscarinic antagonist) were added to an incubation mixture containing 10 nM ^{125}I-alpha-bungarotoxin. The amount of ^{125}I-alpha-bungarotoxin bound was expressed as a percentage of the amount bound in the absence of cholinergic ligands (Fig. 3). The most effective competitor was unlabeled alpha-bungarotoxin, which inhibited labeled toxin binding by 50% (IC_{50}) at a concentration of about 100 nM. Under these experimental conditions in which the concentration of the radiolabeled ligand is much less than the K_D, the IC_{50} provides an estimate of the binding affinity of the unlabeled alpha-bungarotoxin (15). This binding affinity is in close agreement with the K_D determined from the concentration dependency of ^{125}I-alpha-bungarotoxin binding.

It has been demonstrated for the intact *T. californica* acetylcholine receptor that *d*-tubocurarine and alpha-bungarotoxin share a common binding site (4,16). We therefore examined the ability of *d*-tubocurarine to displace ^{125}I-alpha-bungarotoxin binding to the

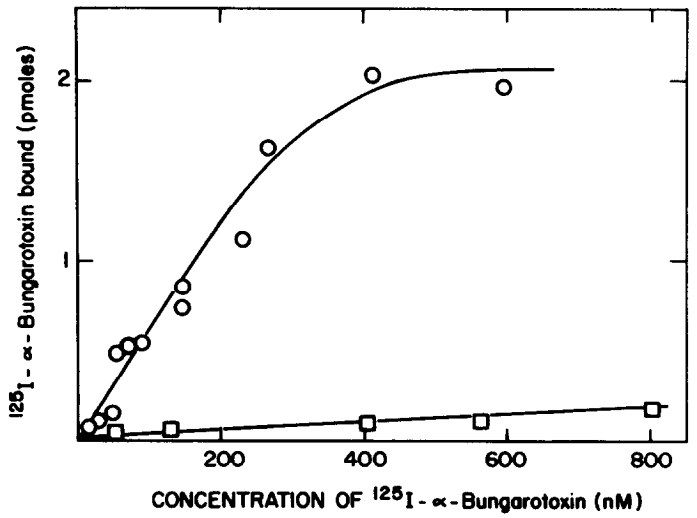

FIG. 2. Concentration dependency of the binding of ^{125}I-alpha-bungarotoxin to the nitrocellulose-bound alpha subunit. Acetylcholine receptor-enriched *T. californica* membrane fragments, containing 4 nmol alpha-bungarotoxin binding sites, in 712 μg of protein were solubilized and applied to a single 8-cm-wide well. Following SDS–gel electrophoresis and transfer to nitrocellulose sheets, sections (1 × 1.4 cm) containing the alpha subunit were excised and incubated for 4 h at 22°C with 600 μl of increasing concentrations of ^{125}I-alpha-bungarotoxin (9 Ci/mmol). ○, ^{125}I-Alpha-bungarotoxin bound to the alpha subunit (corrected for background) as described under Methods. □, Binding in the presence of a 100-fold excess of the unlabeled alpha-bungarotoxin.

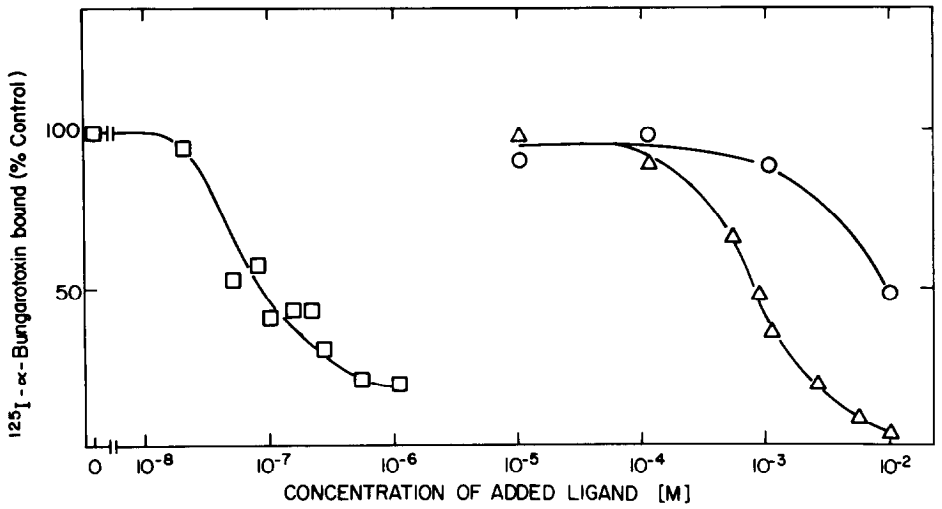

FIG. 3. Concentration dependency of the inhibition of ^{125}I-alpha-bungarotoxin binding by cholinergic ligands. *T. californica* membrane fragments (4.3 nmol alpha-bungarotoxin binding sites in 832 μg of protein) were resolved by SDS-gel electrophoresis and transferred to nitrocellulose sheets (12 × 12 cm) as described under Methods. Sheets were cut into 1-cm-wide strips and incubated for 4 h with 10 nM ^{125}-I-alpha-bungarotoxin and various concentrations of cholinergic ligands. ^{125}I-Alpha-bungarotoxin bound to the alpha subunit was determined as described under Methods and was expressed as percentage of the binding in the absence of added cholinergic ligands. △, *d*-Tubocurarine; ○, atropine; □, unlabeled alpha-bungarotoxin.

alpha subunit when adsorbed on nitrocellulose. Under the experimental condition of low receptor occupancy, *d*-tubocurarine was found to displace 50% of the labeled toxin (IC_{50}) at a concentration of 1 mM. This result suggests that while *d*-tubocurarine most likely binds to the same site as alpha-bungarotoxin on the alpha subunit, it does so with a binding affinity that is reduced by several orders of magnitude when compared to the intact receptor (16). Atropine, on the other hand, is not believed to interact with the nicotinic acetylcholine receptor, and we observed that it does not displace ^{125}I-alpha-bungarotoxin except at very high concentrations. This is most likely a consequence of a nonspecific effect by this ligand.

DISCUSSION

This investigation demonstrates the feasibility of coupling the high resolving power of SDS-gel electrophoresis to a radiolabeled ligand-binding assay by use of the nitrocellulose-gel transfer technique. A mixture of polypeptides obtained from *T. californica* was separated by SDS-gel electrophoresis. They were subsequently transferred without significant loss of resolution to nitrocellulose sheets in an environment designed to remove the SDS and promote renaturation. Following incubation with ^{125}I-alpha-bungarotoxin, a single polypeptide, the alpha subunit retained some of the ligand-binding properties of the native receptor with regard to specificity and saturability. However, this nitrocellulose-bound alpha subunit had about a 1000-fold weaker affinity for alpha-bungarotoxin. The reduced potency of *d*-tubocurarine in displacing the labeled toxin implies that it also binds with a weakened affinity.

The observed reduction in binding affinity might have a variety of explanations. During the transfer process the alpha subunit may not fully renature and consequently may not bind with the high affinity observed for the native

intact receptor. Additionally, the reduced affinity may result from the dissociation of the receptor into its four polypeptide components. High-affinity binding may require the association of all four subunits as in the native receptor. A close association of these polypeptides is suggested by the observation that a photoaffinity derivative of ^{125}I-alpha-bungarotoxin labels all four subunits of the membrane-bound receptor (17). Another possibility is that the alpha subunit may exhibit weak ligand-binding properties because it is adsorbed onto a solid matrix and not in solution. However, Haggerty and Froehner (18) have isolated *T. californica* receptor subunits by preparative SDS–gel electrophoresis, followed by dialysis in a cholate buffer, and have found that only the alpha subunit could bind ^{125}I-alpha-bungarotoxin. The dissociation constant they reported, K_D = 200 nM, is similar to the one in this study. In addition, they found that *d*-tubocurarine inhibited the initial rate of ^{125}I-alpha-bungarotoxin binding with a reduced potency similar to the value estimated by the displacement study reported here. Therefore, the properties of the nitrocellulose-bound alpha subunit closely resemble those of the alpha subunit in solution and it is unlikely that the weaker binding affinity of alpha in this study can be explained by its adsorption onto nitrocellulose sheets.

With the present methodology, 100 ng of the alpha subunit when applied to a lane of a polyacrylamide gel (10 ng/mm^2) is readily detected by autoradiography after a 2-day exposure. Assuming that 100 μg of protein can be resolved within a single gel lane (0.6 cm wide), then 0.1% of the total protein must be the alpha subunit in order to be detected in 2 days. In addition only 1% of the total alpha-bungarotoxin binding sites applied to the gel are recovered on nitrocellulose sheets. Sources of this loss of binding sites include incomplete solubilization of receptor proteins, inefficient protein transfer from the gel to nitrocellulose sheets, renaturation of only some of the alpha subunits, and partial loss of the specific component of binding during the extensive wash procedures. Thus the sensitivity of this assay could be improved by up to 2 orders of magnitude by addressing these problems. A further increase in the sensitivity may be achieved by using ^{125}I-alpha-bungarotoxin radiolabeled to a higher specific activity (up to 2000 Ci/mmol). With an increase in sensitivity the usefulness of this technique could be extended to identify low concentrations of nicotinic acetylcholine receptors from tissues such as mammalian skeletal muscle.

The results presented here have demonstrated the novel use of an analytical technique to study the interaction of a polypeptide ligand, alpha-bungarotoxin, with individual *T. californica* acetylcholine receptor subunits. Since many biologically active polypeptides and peptides have been shown (19–23) to bind with similar affinities to plasma-membrane receptors, the inherent simplicity of this technique suggests its use in the identification of the individual receptor subunits that bind these molecules.

ACKNOWLEDGMENTS

We thank Dr. Don Nelson and Dr. Gary Lancione, Chemistry Department, Clark University, and Trudy Morrison, Microbiology Department, University of Massachusetts Medical School for gifts of affinity-purified *T. californica* acetylcholine receptor and VSV RNA, respectively. We also thank Dr. William Boto, Johns Hopkins Medical School, Department of Immunology, for his helpful suggestions.

Note added in proof. Recently Gershoni *et al.* 1982, (*J. Cell Biol.* **95,** 422a, abstract number 21046) using a technique similar to the one described here, have also found that ^{125}I-alpha-bungarotoxin binds selectively to the alpha subunit with a significantly reduced affinity.

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